

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



USSN 2/250,096
ref 7

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 35/76, C12N 7/00, 7/01, 15/09, 15/85, 15/86		A1	(11) International Publication Number: WO 98/05344 (43) International Publication Date: 12 February 1998 (12.02.98)
(21) International Application Number: PCT/US97/12928 (22) International Filing Date: 3 July 1997 (03.07.97) (30) Priority Data: 08/693,865 5 August 1996 (05.08.96) US 08/814,859 11 March 1997 (11.03.97) US (71) Applicant (for all designated States except US): BRIGHAM AND WOMEN'S HOSPITAL, INC. [US/US]; 75 Francis Street, Boston, MA 02115 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SARKAR, Saumyendra, N. [IN/US]; 102 Queensbury Street, Boston, MA 02115 (US). KUPPER, Thomas, S. [US/US]; 8 Surrey Lane, Weston, MA 02193 (US). DUBIN, Daniel, B. [US/US]; Apartment D-618, 99 Pond Avenue, Brookline, MA 02146 (US). (74) Agent: PLUMER, Elizabeth, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).			(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: BACTERIOPHAGE-MEDIATED GENE THERAPY			
(57) Abstract <p>An improved method for delivering an exogenous gene, e.g., a therapeutic polynucleotide, to a mammalian cell is provided. The method involves using a bacteriophage as a vector to deliver the exogenous gene to a pre-selected target cell. The bacteriophage contains exogenous genetic material that can be transcribed and, optionally, translated in a mammalian cell and includes on its surface a ligand that binds to a receptor on the target cell. The bacteriophage is incapable of injecting the exogenous genetic material into the mammalian cell. The bacteriophages are useful for gene therapy applications and for producing exogenous gene products <i>in vitro</i>.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

BACTERIOPHAGE-MEDIATED GENE THERAPY

Government Support

15 This work was funded in part by the National Institutes of Health under the Grant Numbers AI 25062. The Government may retain certain rights in this invention.

Related Applications

20 This application is a continuation-in-part of U.S. Serial No. 08/814,859, filed March 11, 1989, pending, which is a continuation-in-part of U.S. Serial No. 08/693,865, filed August 5, 1996, pending, the entire contents of which are incorporated herein by reference.

Field Of The Invention

This invention relates to the use of a bacteriophage to express an exogenous gene in a mammalian cell. In particular, the invention relates to the use of bacteriophage vectors for human gene therapy and compositions related thereto.

Background Of The Invention

25 The application of gene therapy for the treatment of human disease has increased steadily since the first human gene therapy trial was conducted in 1989. To date, well over 100 (Science 269: 1050-5 (1995)) gene therapy protocols and clinical trials have been approved by the Recombinant DNA Advisory Committee for the treatment of inherited and acquired diseases. Despite the reported advances in gene therapy technology and the increasing approvals of gene
30 therapy protocols by the National Institutes of Health, the delivery and long-term expression of exogenous genes in specific tissues for the treatment of genetic disease remains a formidable challenge.

Vectors which are being studied in gene therapy trials include retroviruses, adenoviruses, adeno-associated virus, plasmids and liposomes. Each has significant limitations. Although the
35 adenovirus reportedly is a relatively efficient gene delivery vehicle for both dividing and non-dividing cells, its drawbacks include (1) limitation of inserted expression cassette to seven kilobases, (2) induction of inflammation in mammalian hosts, (3) toxicity to target cells when administered in high titers, (4) inability to target specific cell types, and (5) transient expression in infected cells. Moreover, both injection of fully packaged adenovirus and subsequent
40 expression of adenoviral antigenic proteins in infected cells provoke a host immune response that may significantly limit the bioavailability of repeated administration of the vector. Indeed, an inflammatory response to adenovirus during a cystic fibrosis gene therapy trial has been reported.

As an alternative to adenovirus, adenoassociated virus has been proposed as an

alternative to adenovirus, and does have certain advantages, including a preferential site of chromosomal integration. Although the adeno-associated virus has not been associated with inflammation, this virus' genome is only 4.7 kilobases in size and can accommodate inserts of up to 5 kb in size. Thus, its primary limitation is in the size of the "expression cassette" that it can deliver to the target cell. An "expression cassette" is a term of art that refers to an inserted exogenous DNA that optionally contains transcriptional regulatory sequences, translational regulatory sequences, coding sequences, or intervening sequences necessary for efficient expression of the included coding sequence. The cost of manufacturing adeno-associated virus is high compared to that of adenovirus. Furthermore, adeno-associated virus is difficult to grow at high titer and cannot be targeted to defined cellular targets.

The majority of gene therapy trials employ retroviral vectors for delivering an exogenous gene into mammalian cells and are susceptible to complement-mediated destruction. However, currently utilized retroviral vectors can transduce only dividing mammalian cells. In addition, the retroviruses can accommodate inserts up to only seven kilobases, thereby limiting the size of exogenous genetic material that can be delivered using this type of vector. In addition, the cost of manufacturing retroviral vectors has been estimated to be approximately \$100,000 per lot, reportedly due to the high costs associated with producing high titers of recombinant retroviral vectors in animal cells (D. Holzman, "Gene Therapy Depends on Finding the Right Vector", JNCI, Issue 6, vol. 87 (1995) NEWS pg 406). As late as 1995, only about half of the 76 retroviral clinical trials (Science 269: 1050-5 (1995)) approved for gene therapy had been initiated, in part, due to the high expense and difficulty in undertaking the manufacture of the retroviral vectors. The concern that retroviruses may replicate *in vivo* has inhibited clinical acceptance of retrovirus-mediated gene therapy and at least one incidence of replication of a retroviral vector occurring in clinical materials has been reported (D. Holzman, *ibid.*). There is also concern that random integration of retroviral vectors could disrupt or otherwise adversely affect host cell gene expression.

Non-viral vectors for gene therapy that are being studied in clinical trials include liposomes and naked DNA delivery systems. Low efficiency of transfection hampers both of these methods, neither of which permit targetable delivery to specific cell types. In addition, liposomes can be toxic to cells.

Despite the wide range of vectors currently available for human gene therapy, it is generally agreed that a clinically efficient and cost-effective vector for delivery of an exogenous

gene to specific mammalian cells or tissues has not been identified. The inadequacies of the above-identified vectors include: (1) induction of an immune response in the host; (2) possible deleterious recombination events; (3) limitations in the size of the expression cassette that can be inserted into the vector; (4) lack of specificity of the vector for delivering the expression cassette to a particular target cell; (5) inability to target a vector to both dividing and non-dividing cells; (6) high vector production cost, (7) low efficiency of transfection (non-viral vectors), (8) unacceptable toxicity, (9) low efficiency of expression *in vivo*, and (10) transience of expression of the exogenous genetic material. Accordingly it is incumbent upon scientists interested in realizing the unfulfilled promise of gene therapy to develop vectors that can overcome these shortcomings.

Summary Of The Invention

Applicants have discovered that certain well-characterized bacteriophages can be modified to contain exogenous genetic material that can be transcribed and, optionally, translated in a mammalian cell. Moreover, modification of these bacteriophages to include specific ligands and gene transcription regulatory elements permits control over the cell(s) to which the expression cassette is delivered and in which the delivered genetic material will be expressed. Furthermore, these bacteriophages can be modified to include nuclear localization and endosomal lysis signals to enhance the efficiency of transfection. Despite genetic modification, these bacteriophages preferably maintain the ability to propagate to high titer in a prokaryotic host, thus greatly facilitating production.

Thus, the invention provides compositions and methods for using these bacteriophages to introduce an exogenous polynucleotide (e.g., a therapeutic polynucleotide) into a pre-selected target cell via receptor-mediated delivery. According to one aspect of the invention, the bacteriophages are used to deliver a therapeutic polynucleotide into a mammalian cell for human gene therapy. In yet another aspect of the invention, the bacteriophages are used to deliver an exogenous polynucleotide into a mammalian cell for the production, *in vitro* or *in vivo*, of a polynucleotide transcription or translation product. According to yet other aspects of the invention, compositions containing the bacteriophages of the invention and methods for preparing and using the same to produce exogenous polynucleotide transcription and translation products *in vitro* also are provided. In certain preferred embodiments of the foregoing aspects of the invention, the bacteriophage contains exogenous genetic material that can be both transcribed and translated in a mammalian cell. In other preferred embodiments, the bacteriophage contains

exogenous genetic material that is transcribed, but not translated, in a mammalian cell (e.g., ribozyme and antisense constructs). Exemplary bacteriophages which can be modified to satisfy this criteria are provided in Table 1. (Tables 1-8 are presented at the end of the detailed description of the invention, immediately preceding the specific Examples section.)

Advantageously, these bacteriophages can be propagated in prokaryotic cell(s), thereby permitting the large scale production of the bacteriophages of the invention at a relatively low cost in comparison with other gene therapy vectors. Furthermore, since the bacteriophage structural genes are controlled by prokaryotic promoters, no transcription of native bacteriophage genes will occur in the mammalian host cell. Therefore, cells transfected with a modified bacteriophage will not express foreign proteins on their cell surface and, thus, will not induce cell-mediated immunity.

The preferred bacteriophages of the invention include the lambda and p1 phages. These preferred phages, after targeting modifications, have in common the ability to deliver to a mammalian cell(s) a therapeutic polynucleotide that is between about 1-100 kilobases. In particular, the preferred phages have in common the ability to deliver to a mammalian cell(s) a therapeutic polynucleotide of large size: between about 9-23 kilobases for lambda Dash II and between about 75-100 kilobases for p1. The preferred modified lambda phages of the invention can accommodate between about 9-50 kilobases. Thus, the invention is particularly useful for delivering to a target cell, an exogenous polynucleotide expression cassette (e.g., a therapeutic polynucleotide) containing a single gene or multi-gene complexes that are greater than 9 kilobases in length, something that cannot be accomplished by currently available viral vectors.

According to one aspect of the invention, an improved method for gene therapy is provided. The improvement lies in using a bacteriophage to deliver a therapeutic polynucleotide to a target cell in a mammalian recipient. In particular, the invention provides, for the first time, a viral vector that delivers to a target cell, an exogenous polynucleotide containing up to 9 to 23 kilobases for lambda Dash II, up to 50 kilobases for lambda-derived cassettes containing only COS sequences and no packaging protein coding sequences (minimal lambda cassette), and up to 75 to 100 kilobases for p1. The bacteriophages of the invention contain on their surfaces ligands to achieve delivery of the bacteriophages into a pre-selected target cell. The pre-selected target cell contains on its surface a receptor that selectively binds to the ligand, forming a ligand-receptor complex that is internalized by the cell. Although Applicants do not wish to limit the scope of the invention to a particular mechanism, it is believed that formation of the ligand-

receptor complex induces internalization of the complex via a receptor-mediated delivery mechanism such as that utilized by native eukaryotic viruses (e.g., adenovirus) and as that described in U.S. Patent No. 5,108,921, issued to Low, et al. and U.S. Patent No. 5,166,320, issued to Wu, et al., internalization of the bacteriophage into the target cell (i.e., transfection) can be performed *in vivo* or *ex vivo*. According to another aspect of the invention the bacteriophage vector further contains a nuclear localization signal (e.g., retrovirus) (Naldini et al. Science 1996; 272:263) as well as an endosomal lysis signal (e.g., adenovirus) (Wagner et al. Proc Natl Acad Sci 1992;89:6009) which, alone or in combination, enhance the expression of the delivered polynucleotide. Alternatively, chloroquine or other inhibitors of lysosomal/endosomal enzymatic catabolism can be co-administered with the vector. The use of nuclear localization signal(s), endosomal lysis signal(s), and/or endosomal enzyme inhibitors optimize expression of the delivered polynucleotide by minimizing the likelihood of DNA degradation that may occur as the vector traffics to the nucleus. In the particularly preferred embodiments, the delivered polynucleotide is designed and constructed in accordance with standard practice to integrate into the target cell chromosome. Alternatively, the vector may be maintained episomally in the cell. Accordingly, the novel bacteriophages disclosed herein are useful for *in vivo* and *ex vivo* gene therapy, as well as for producing in culture or in animals, gene products of the therapeutic polynucleotides (e.g., transcription products such as antisense RNA or catalytic RNA (e.g., ribozyme), and translation products such as peptides and proteins).

The improved method for gene therapy is useful for introducing a therapeutic polynucleotide (e.g., a polynucleotide for treating or diagnosing a medical condition) into a target cell of a mammalian recipient. The recipient is diagnosed as having a medical condition that is treatable by administration to the recipient of the therapeutic polynucleotide or a product thereof (e.g., a transcription product such as an antisense RNA or a translation product such a peptide or protein). The improved gene therapy method involves: (1) contacting the bacteriophage with the target cell under conditions (a) to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and (b) to allow the bacteriophage to enter the target cell; and (2) allowing the target cell to live under conditions such that the therapeutic polynucleotide is transcribed therein. Exemplary medical conditions and their respective therapeutic polynucleotides (or therapeutic polynucleotide products) that are useful for treating these medical conditions are provided in Table 2 (see, also, BioWorld Financial Watch, Monday Sept. 19, 1994, pp4-10, American Health Consultants, Inc.; Gene

Therapy A Primer for Physicians, Culver KW, Ed. 1996, Mary Ann Liebert, Inc. New York).

Therapeutic polynucleotides that are particularly suited for delivery using the bacteriophages of the invention are provided in Table 3. Exemplary target cells to which the bacteriophages can deliver these and other therapeutic polynucleotides are provided in Table 4. The preferred target cells express on their surfaces receptors that mediate cellular uptake of the bacteriophage vector via receptor-mediated endocytosis. More preferably, the target cells are non-phagocytic.

Screening methods for identifying target cells that internalize the bacteriophage vectors by way of receptor-mediated endocytosis are disclosed in the Examples. In general, these methods assay uptake by the target cell of the bacteriophage vector in the presence or absence of one or more known inhibitors of receptor-mediated endocytosis. The invention is directed to bacteriophage vectors that enter the cell via receptor-mediated endocytosis and not by the natural phage prokaryotic transduction pathway. Accordingly, in the preferred vectors, the bacteriophage tail proteins that are required for natural phage transduction are either, absent, non-functional in a prokaryotic host, or not capable of mediating injection of genetic material into a eukaryotic host cell. The screening methods disclosed in the Examples permit the selection of bacteriophage vectors and target cells which satisfy the above-noted criteria.

Advantageously, the preferred bacteriophage vectors are abortive to lytic growth in the natural bacterial flora of the mammalian host. Such modified bacteriophage vectors are also abortive to lytic growth in Su^0 (natural E. Coli host without the amber suppressor gene mutation $supE$ or $supF$, both of which code for tRNAs) bacterial host strains in vitro. In the preferred embodiments, the bacteriophage vector genome is modified so as to contain an amber mutation inserted in-frame into a bacteriophage tail protein gene, e.g. J, M, or H gene, such that in an Su^0 bacterial host the tail protein gene is truncated and non-functional and, hence, abortive to lytic growth. Advantageously, the bacteriophage vectors with tail protein amber mutations propagate only in E. Coli strains containing either the amber suppressor gene mutation $supE$ or $supF$.

Alternatively, the bacteriophage vectors have temperature-sensitive tail protein mutations abortive to lytic growth at mammalian host physiologic temperature. At temperatures other than (greater or less than) the mammalian host physiologic temperatures, the tail proteins are expressed and function normally and can mediate prokaryotic cell infections in vitro. Accordingly, such modified bacteriophage vectors are packaged in vitro in cell free systems using purified protein packaging extracts and engineered cosmid vectors. In the preferred embodiments, the bacteriophage vectors have temperature-sensitive tail protein mutations such

that at temperatures other than (greater or less than) the mammalian host physiologic temperature, the tail proteins are expressed and function normally and can mediate prokaryotic cell infections; however, at mammalian physiologic temperature, the temperature sensitive mutation inactivates the wild type tail function.

In the preferred embodiments, the bacteriophage genome includes a promoter to control transcription and/or translation of the therapeutic polynucleotide in the target cell. Exemplary promoters are provided in Table 5. More preferably, the promoters are cell or tissue specific (i.e., they are functional only in particular types of cells or tissues), thereby providing an additional means for controlling expression (i.e., transcription and translation) of the therapeutic polynucleotide in the target cell. Optionally, the bacteriophage genome includes additional regulatory sequences, e.g., enhancers, for further controlling expression of the therapeutic polynucleotide in the target cell. Exemplary enhancers also are provided in Table 5. In the preferred embodiments, the enhancers are target cell specific. Optionally, the bacteriophage genome can include eukaryotic origins of replication (e.g., from mammalian chromosomes), telomeres and centromeres to permit autonomous replication of the bacteriophage genome within the target cell and segregation of the replicated genome into the target cell progeny.

The bacteriophage of the invention includes on its surface a ligand which selectively binds to a receptor on the target cell surface to form a ligand-receptor complex. The complex is internalized by the target cell, presumably by receptor-mediated endocytosis. Applicants do not intend to limit the invention to a particular internalization mechanism, other than limiting the invention to exclude the natural phage transduction pathway that, as described in the literature, involves tail protein mediated injection of the bacteriophage genome into the target cell. The ligand can be attached to the surface of the bacteriophage using, for example, chemical modification methods, (e.g., galactosylation), genetic engineering methods (e.g., inserting a sequence encoding the ligand into the bacteriophage genome, in frame, such that the ligand is expressed on the surface of the bacteriophage), specific adsorption (e.g., coating an antibody onto the surface of a bacteriophage) or a combination of genetic engineering and affinity binding methods (e.g., expressing avidin on the bacteriophage surface to form an "avidin-labeled bacteriophage" and binding a biotinylated ligand thereto). Such avidin-labeled bacteriophages also are useful as intermediates in attaching virtually any ligand to the surface of a bacteriophage, provided that following biotinylation, the ligand retains its functional activity (i.e., the ability to selectively bind to its receptor to form a ligand-receptor complex). Potential ligands include

peptide or nucleotide polymers, macromolecular aggregates, such as lipoproteins or any chemical structure, either naturally occurring, synthesized, or generated by combinatorial chemistry techniques. Exemplary receptors and preferred ligands for targeting specific cell types are provided in Table 6. The preferred ligands include: low density lipoprotein (apoprotein B100), very low density lipoprotein (apoprotein E or a single chain variable immunoglobulin gene fragment that has high affinity for the VLDL receptor), HDL (apoA1), galactose, c kit ligand, transferrin, insulin, heregulin, and RGD or RGD-containing polypeptides (cyclic RGD).

The invention also embraces ligand-labeled bacteriophages in which the ligand is an antibody (or fragment thereof) that selectively binds to an antibody receptor (e.g., an Fc receptor) on the surface of a target cell. The antibody can be attached to the surface of the bacteriophage by, for example, selectively binding an antibody to a bacteriophage surface antigen. Thus, the invention is useful for targeting the delivery of an exogenous polynucleotide to virtually any Fc receptor bearing target cell *in vivo* or *ex vivo* via Fc receptor-mediated internalization. Finally, the avidin labeled phage can be bound to a biotinylated antibody. This antibody can bind to its cognate antigen on the cell surface, an event that is followed by patching, capping, and internalization. Optionally, the bacteriophage is modified, after *in vitro* propagation and packaging, to prevent or reduce the likelihood that the bacteriophage will enter bacteria that may be endogenous to the mammalian recipient or that may be a contaminant of target cells in culture. Such modifications can take the form of attaching the ligand to those portions of the bacteriophage (e.g., the "D" protein of the phage head) in a manner to inhibit the ability of the bacteriophage to attach to and/or penetrate its bacterial host. Alternatively, the ligand can be attached to the surface of a bacteriophage at a location that is not involved in phage attachment or penetration of the bacterial host. Other procedures, such as genetic engineering/chemical modification, can be used to interfere with the function of one or more bacteriophage encoded translation products that are essential for phage attachment and/or penetration into the bacterial host. (See, e.g., C. Merrill, et al., "Long-circulating bacteriophage as antibacterial agents", PNAS USA 93:3188-3192 (1996), which describes the importance played by the amino acid glutamic acid at position 158 of the capsid E protein in the insertion, *in vivo*, of bacteriophage lambda into *E. coli*).

According to yet another aspect of the invention, an avidin-labeled bacteriophage is provided. The bacteriophage has a genome in which the recombinant exogenous genetic material can be transcribed and translated in a mammalian cell. Since the native bacteriophage coding

sequences are regulated by prokaryotic promoters, in the ensuing discussion, the recombinant bacteriophage genome will refer only to the inserted genetic material whose expression is controlled by eukaryotic regulatory elements. Preferably, for bacteriophage vectors that are intended to produce a polypeptide, the recombinant bacteriophage genome can be both transcribed and translated in the mammalian cell. As mentioned above, the avidin-labeled bacteriophages are useful as intermediates in generating the ligand-labeled bacteriophages of the invention. Alternative high affinity binding pairs can be substituted for the avidin/biotin binding pair in accordance with the methods of the invention. For example, streptavidin can be substituted for avidin to form a streptavidin-labeled bacteriophage that can be allowed to bind to a biotinylated ligand to form a streptavidin-biotin complex. Alternatively, modified avidin or streptavidin may be employed.

According to yet another aspect of the invention, a method for introducing an exogenous polynucleotide into a target cell, preferably, a human cell, is provided. The method involves: (1) contacting the bacteriophage with the target cell under conditions: (a) to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and (b) to allow the bacteriophage to enter the target cell; and (2) allowing the target cell to live under conditions such that the exogenous polynucleotide is transcribed and, optionally, translated therein. As used herein, the phrase "exogenous polynucleotide" refers to a nucleic acid that is not normally present in the naturally-occurring (i.e., non-recombinant) bacteriophage. Thus, exogenous polynucleotides embrace the above-described therapeutic polynucleotides, as well as polynucleotides that are not intended for therapeutic applications (e.g., polynucleotides that encode a mammalian protein or protein complex for production of the protein or protein complex in cell culture, transcription regulatory elements, telomeres, centromeres, splice junctions, autonomous replicating sequences, recombination specific sequences). Exemplary bacteriophages and ligands that are useful in accordance with this method are described above in reference to the improved method for gene therapy. According to a particularly preferred embodiment, the method further involves the step of isolating an exogenous polynucleotide product (e.g., a transcription or translation product) from the target cell.

According to yet another aspect of the invention, a bacteriophage that is useful for practicing the above-described methods for delivering an exogenous polynucleotide (e.g., a therapeutic polynucleotide) to a target cell is provided. The bacteriophage, which can be propagated in prokaryotes, contains a recombinant genome that can be transcribed and,

optionally, translated in a mammalian cell. More preferably, the recombinant bacteriophage genome can be both transcribed and translated in the mammalian cell. The surface of the bacteriophage is modified to contain thereon a ligand that selectively binds to a receptor on the mammalian target cell.

5 The bacteriophages of the invention are useful in the preparation of a medicament for treating a medical condition that is treatable by administration to the mammalian recipient of the therapeutic polynucleotide or a product thereof. The bacteriophages can be placed in a pharmaceutically acceptable carrier to form a pharmaceutical composition which can be administered to the recipient in accordance with standard clinical practice known to one of
10 ordinary skill in the art. In a particular embodiment, the pharmaceutical composition is contained in an implant that is suitable for implantation in the mammalian recipient. Thus, the methods and compositions of the invention provide for an implantable bacteriophage gene therapy vector that is useful for delivering a therapeutic polynucleotide to the mammalian recipient over an extended period of time.

15 According to another aspect of the invention, a kit is provided which contains: (1) a first container containing an encapsidated bacteriophage having an appropriate surface marker and a genome (preferably, a genome having multiple cloning sites, such as the lambda DASH II genome) and instructions for inserting exogenous genetic material, which may contain coding sequence and upstream and downstream regulatory elements into the genome, preferable into the
20 multiple cloning site, (2) a second container containing an agent for attaching a ligand to the surface of the bacteriophage, wherein the ligand is designed to bind to a receptor on the surface of a mammalian cell; and (3) instructions for attaching the agent to the surface of the bacteriophage. More preferably, the kit further includes instructions for transducing a desired target mammalian cell. Alternatively, the desired ligand coding sequence is included in the
25 genome of the bacteriophage and the agent for attaching the ligand to the surface is unnecessary.

 These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments and to the accompanying drawings.

 All references, patent publications and patents identified in this disclosure are
30 incorporated in their entirety herein by reference.

Brief Description of the Drawings

Fig. 1, including 1A, 1B and 1C, is a schematic diagram describing the experimental strategy for generation of targeted bacteriophage vectors by chemical modification and use of the modified bacteriophage for gene delivery to cells of specific mammalian tissue origin.

Fig. 2 is a schematic diagram describing the experimental strategy for generation of targeted bacteriophage vectors by fusing the coding DNA sequences of a ligand "L" into the bacteriophage virion capsid specific "D" gene.

Fig. 3 is a schematic representation of a chimera of lambda DASH II /CMV promoter enhancer/beta-galactosidase gene sequences. Fig. 3A shows a restriction enzyme map and schematic representation of a restriction digest of the bacteriophage vector. Fig. 3B shows a schematic representation of the CMV promoter enhancer/beta-galactosidase gene sequences and ligation of this DNA to the digested bacteriophage to form the chimera, followed by (a) in vitro packaging, (b) propagation in *E. coli*, and (c) purification by CsCl gradient centrifugation to form a bacteriophage that is capable of expressing the beta-galactosidase (beta-gal) gene under the control of the CMV promoter in mammalian cells. The beta-gal gene contains a nuclear localization signal that directs localization of the translation product to the nucleus.

Fig. 4 is a schematic representation of a chimera of lambda DASH II/PGK promoter/-galactosidase gene sequences that are formed as described above in Fig. 3.

Fig. 5 is a schematic representation of a MCK/DMD/lambda bacteriophage chimeric DNA construct.

Fig. 6 is a schematic representation of a heregulin/lambda bacteriophage chimeric DNA construct in which a portion of the heregulin cDNA is fused, in frame, with the 3' end of the wild type capsid D-gene. Fig. 6A shows generation of the polynucleotide fragments; Fig. 6B shows the joining of the polynucleotide fragments; and Fig. 6C shows the generation of a modified bacteriophage expressing the heregulin-protein D chimeric capsid genes.

Fig. 7 is a schematic representation of A) the generation of targeted bacteriophage vectors by fusing a cyclic RGD ligand onto the bacteriophage lambda virion head specific D-gene product; B) generation of fragments for gene fusion; C) joining the fragments; D) generation of targeted lambda phage vector expressing the cyclic RGD-D chimeric capsid and containing the CMV beta-gal reporter gene; E) generation of cyc RGD modified lambda DASH II bacteriophage containing the murine dystrophin gene expression cassette; and F) generation of cyc RGD modified lambda DASH II bacteriophage containing the Factor VIII/IRES/Von

Willebrand's Factor gene expression cassette. In reference to fig. 7B, the primers are defined as follows: primer "a" (SEQ. ID NO. 18) contains only wt sequence of the lambda DASH II "C" gene; primer "b" (SEQ. ID NO. 19) has a 3' end that is complementary to the 3' end of the wt "D" gene and a 5' end which contains the coding sequence to cyclic RGD; primer "c" (SEQ. ID NO. 20) has a 5' end that is complementary to the 5' end of cyclic RGD and a 3' end which is complementary to the 5' end of the wt "E" gene; and primer "d" (SEQ. ID NO. 21) contains only wt sequence of the lambda DASH II "E" gene. In reference to fig. 7D, the preparation of the lambda DASH II/CMV beta-gal vector was by first digesting the vector with BsrGI and Eco R1 (*lambda coordinates) and cleaving into fragments. Fragment #2 (5220-6142bp) was then separated and removed by gel electrophoresis. Co-digestion with Eco R1 favors recombination of productive phage genome. (Fragment 1: *1-5220; Fragment 2: *5221-*6142; Fragment 3: *6143-*15855; Fragment 4: *15856-CMV-betagal-*41900; and Fragment 5: *41900-48000).

Detailed Description of the Invention

The instant disclosure provides bacteriophages for delivering an exogenous polynucleotide into a target cell, preferably a mammalian cell. A "bacteriophage", for the purposes of this invention, refers to a bacteriophage that: (1) contains exogenous genetic material that can be transcribed and, optionally, translated in a mammalian cell and (2) contains on its surface a ligand that selectively binds to a receptor on the surface of a target cell, such as a mammalian cell. As used herein, "exogenous genetic material" refers to a polynucleotide (e.g., nucleic acid or oligonucleotide), either natural or synthetic, that is not naturally found in a bacteriophage, or if it is naturally found in the bacteriophage, it is not transcribed or expressed at biologically significant levels by the bacteriophage. "Exogenous genetic material" includes a non-naturally occurring polynucleotide that can be transcribed into an anti-sense RNA, as well as all or part of a "heterologous gene" (i.e., a gene encoding a protein which is not expressed or is expressed at biologically insignificant levels in a naturally-occurring bacteriophage). Thus, for example, the instant invention embraces the introduction into a mammalian cell of an expression cassette including a recombinant gene containing an inducible promoter operably coupled to a coding sequence of a therapeutic polynucleotide. In the preferred embodiments, the exogenous genetic material of the bacteriophage can be both transcribed and translated in the mammalian target cell. Exemplary bacteriophages that satisfy at least the first of these criteria are provided in Table 1. Exemplary ligands that can be attached to the bacteriophage surface, e.g., covalently coupled to the surface, expressed, or specifically adsorbed or affinity bound thereto, are provided in Table 6. (Tables 1-8 are presented at the end of the detailed description of the invention, immediately preceding the specific Examples section.)

The bacteriophages are useful for delivering an exogenous polynucleotide into a mammalian target cell for *ex vivo* and *in vivo* gene therapy, as well as for producing exogenous polynucleotide products (e.g., transcription products such as antisense mRNA or catalytic RNAs and translation products) in culture or *in vivo*. The bacteriophages of the invention are particularly useful for delivering an exogenous polynucleotide containing between about one and one-hundred kilobases to a mammalian target cell, depending on the particular bacteriophage that is selected. For example, lambda and p1 can be used to deliver exogenous polynucleotides containing up to 9 to 23 kb and up to 75 to 100 kb, respectively. A minimal lambda cassette can deliver exogenous polynucleotides containing up to 50 kb. In contrast, conventional viral vectors for gene therapy viral vectors can accommodate, at best, a polynucleotide containing up to about

seven kilobases for delivery to a mammalian cell. Thus, the instant invention advantageously provides a method for delivering relatively large genes and/or multi-gene complexes to a mammalian cell for gene therapy purposes and for the *in vitro* or *in vivo* production of gene products. In general, the bacteriophages of the invention can be propagated in prokaryotic cells. Accordingly, the cost of producing the bacteriophages of the invention is relatively inexpensive compared to the cost of producing more conventional gene therapy vectors, such as retroviruses, adenovirus, or adeno-associated virus.

According to one aspect of the invention, an improved method for gene therapy is provided. The improved gene therapy method utilizes a bacteriophage as a vector to introduce a therapeutic polynucleotide into a target cell of a mammalian recipient. The improved gene therapy method involves two steps: (1) contacting the bacteriophage with the target cell under conditions (a) to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and (b) to allow the bacteriophage to enter the target cell; and (2) allowing the target cell to live under conditions such that the therapeutic polynucleotide is transcribed therein. The mammalian recipient is diagnosed as having a medical condition that is treatable by administration to the recipient of the therapeutic polynucleotide or a product thereof. In the preferred embodiments, the mammalian recipient is a human. Exemplary medical conditions and their respective therapeutic polynucleotides (or products thereof) that are useful for treating these conditions are provided in Table 2. In general, the medical conditions that are treatable in accordance with the methods of the invention include genetic diseases (i.e., diseases that are attributable to one or more gene defects) and acquired pathologies (i.e., pathological condition that are not attributable to an inborn genetic defect). The improved method for gene therapy also embraces prophylactic processes (i.e., delaying the onset of the foregoing medical conditions).

The bacteriophage genome contains a therapeutic polynucleotide that encodes a therapeutic polynucleotide product which is useful for treating (i.e., delaying the onset, inhibiting or reducing the symptoms of) the medical condition. As used herein, a "therapeutic polynucleotide" refers to a polynucleotide that mediates a therapeutic benefit in a recipient of the polynucleotide or product thereof. A therapeutic benefit may be an alteration of cell proliferation, a change of expression of a single or multiple genes or proteins, a cytotoxic effect against a pathogen, inhibition of viral replication, replacement of a defective gene and the like. Therapeutic polynucleotides may be administered in the form of a polynucleotide operably

joined to regulatory sequences, disposed in the bacteriophage vector for replication or regulated expression, or in separate non-operable pieces that can become operably joined in the target cell to yield an operable expression system. Therapeutic polynucleotides include genes encoding the transcription and translation products identified in Table 2. Therapeutic polynucleotides also embrace polynucleotides that encode diagnostic agents that can be detected *in situ* or *ex vivo* and that are useful in diagnosing a medical condition. Therapeutic polynucleotides that encode diagnostic agents include the genes encoding, for example, an enzyme that catalyzes a reaction, *in situ*, to yield a detectable product. Thus, as used herein, a "therapeutic polynucleotide product" refers to a molecule produced as a result of transcription or translation of the therapeutic polynucleotide. Therapeutic polynucleotide products include transcription products (e.g., antisense mRNA and catalytic RNA) and translation products (e.g., proteins or peptides) of the therapeutic polynucleotide.

Antisense oligonucleotides that have been approved for gene therapy protocols and/or clinical trials are provided in Table 2. As used herein, the phrases "antisense oligonucleotides" or "antisense" describe an oligoribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an RNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of the mRNA. The antisense molecules are designed so as to hybridize with the target gene or target gene product and thereby, interfere with transcription or translation of the target mammalian cell gene. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon the known sequence of a gene that is targeted for inhibition by antisense hybridization, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 7 and, more preferably, at least 15 consecutive bases which are complementary to the target. Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are

antisense to any region of the gene or RNA (e.g., mRNA) transcripts, in preferred embodiments the antisense oligonucleotides are complementary to 5' sites, such as translation initiation, transcription initiation or promoter sites, that are upstream of the gene that is targeted for inhibition by the antisense oligonucleotides. In addition, 3'-untranslated regions or telomerase binding sites may be targeted. Furthermore, 5' or 3' enhancers may be targeted. Targeting to mRNA splice sites has also been used in the art. In at least some embodiments, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., Cell Mol. Neurobiol. 14(5):439-457 (1994)) and at which proteins are not expected to bind. The bacteriophages of the invention, by virtue of their ability to accommodate therapeutic polynucleotides which are relatively large in size, are particularly useful for delivering to the target cell a polynucleotide that encodes multiple copies of the same or different mRNAs in tandem, thereby increasing the effective concentration of antisense mRNA in the target cell.

The selective binding of the antisense oligonucleotide to a mammalian target cell nucleic acid effectively decreases or eliminates the transcription or translation of the mammalian target cell nucleic acid molecule. Reduction in transcription or translation of the nucleic acid molecule is desirable in those medical conditions where transcription and translation of the mammalian target cell nucleic acid leads to an adverse medical condition. For example, the antisense oligonucleotides of the invention can be used to reduce the expression of oncogenes to treat cancers whose proliferation is mediated by expression of these oncogenes.

The bacteriophages of the invention are also useful for delivering therapeutic polynucleotides that encode specific antigen peptides to antigen presenting cells for processing and presentation at the cell surface to enhance the immune system response of the mammalian recipient to a specific peptide antigen. Exemplary peptide antigens that can be expressed to induce or otherwise enhance an immune response are shown in Table 7. In the preferred embodiments, the therapeutic polynucleotide encodes one or more peptide antigens that vaccinate the mammalian recipient against a tumor, a virus, a bacteria, or a parasite. Optionally, auxiliary therapeutic polynucleotides are inserted into the bacteriophage genome to enhance or otherwise improve the therapeutic efficacy of the therapeutic polynucleotide product in treating the condition. Exemplary auxiliary polynucleotides for delivery to the mammalian target cell include polynucleotides encoding tumor suppressor genes, polynucleotides encoding antisense mRNA or encoding catalytic RNA that inactivate oncogenes, and polynucleotides that render a

target tumor cell more susceptible to an administered drug (e.g., suicide genes encoding, for example, thymidine kinase). Auxiliary polynucleotides also include polynucleotides encoding cytokines that enhance a naturally occurring anti-tumor immunity. Exemplary cytokines which have this function include, e.g., IL-4, TNF, IL-2, and GM-CSF.

The therapeutic polynucleotide is inserted into the bacteriophage genome using conventional recombinant DNA techniques. See, e.g., *Methods in Enzymology*, vol. 152, ed. S. L. Berger, A.R. Kimmel (1987) Academic Press, New York, NY. In the preferred embodiments, the bacteriophage is a lambda phage and the therapeutic polynucleotide is inserted into well-defined restrictions sites in the lambda phage. (See, e.g., the Examples and figures.) Optionally, recombination sequences (i.e., polynucleotides having a nucleic acid that allows homologous recombination) are provided at the 5' and 3' ends of the therapeutic polynucleotide to permit site-directed insertion of the therapeutic polynucleotide into a preselected location in the genomic DNA of the target cell via homologous recombination.

In contrast to the viral vectors that presently are available for human gene therapy, the bacteriophages of the invention can accommodate a therapeutic polynucleotide containing between about one and up to one-hundred kilobases, depending upon the particular bacteriophage selected. For example, lambda bacteriophages, e.g. lambda DASH II, and p1 phage can accommodate up to about 9 to 23 kb and up to about 75 to 100 kb, respectively. Minimal lambda cassette, such as described below, can accommodate up to about 50 kb. Preferably, the therapeutic polynucleotide contains between about 10 and 90 kilobases, more preferably, the therapeutic polynucleotide contains between about 15 and 85 kilobases. Thus, the improved gene therapy method disclosed herein is particularly useful for gene therapy applications which require administration of a single therapeutic polynucleotide (or a product thereof) having a size within the foregoing kilobase range, as well as for delivering multiple therapeutic polynucleotides which, together, have a size within this kilobase range. Exemplary therapeutic polynucleotides containing more than 7 kilobases include dystrophin, members of the globin gene complex, clotting factor VIII, von Willebrand's factor, collagen type VII, fibrillin, and any other gene(s)/gene complexes than are too large to deliver (efficiently) to mammalian cells using conventional viral vectors. Additional therapeutic polynucleotides that can be delivered in accordance with the methods of the invention and that fall within the preferred kilobase size ranges can be identified by, for example, referring to the GenBank or other gene sequence data bases. See, also, Table 3 for a list of preferred therapeutic polynucleotides that can

be delivered using the bacteriophages disclosed herein. The delivery of a therapeutic polynucleotide containing more than 7 kilobases has not been possible using conventional gene therapy viral vectors. A summary of the insert size limitations for conventional gene therapy vectors compared to the bacteriophages disclosed herein is provided in Table 8.

5 The packaging and engineering of the lambda bacteriophage vector can be modified to permit the vector to accommodate up to approximately 50 kb of exogenous coding sequence. This approach involves engineering a recombinant cosmid vector DNA construct that contains an antibiotic resistance gene, e.g. ampicillin, a lambda origin of replication, and a DNA insert up to 50 kb in size flanked by COS (Cohesive ends of wild type bacteriophage lambda genome) sites.
10 This cosmid can be replicated to very high copy numbers in standard strains of E. Coli and then can be isolated using standard techniques for use in the packaging as described below.

The second component of this modified packaging system is a COS-negative lambda lysogen strain of bacteria in whose bacterial chromosome is integrated the structural proteins and enzymes requisite for packaging of an infective lambda virus. Using standard recombinant
15 techniques (Molecular Cloning, 2nd Edition, Sambrook et al., Cold Spring Harbor Laboratory, 1989), the lysogen strain is engineered to contain modifications of certain packaging proteins such that the final modified lambda phage vector is able to effectively target the intended cell type(s), sub-cellular compartments, or sub-cellular organelles. For example, E. Coli can be infected with a modified bacteriophage that contains a fusion D gene-RGD construct. Using
20 standard methods lysogen that contain the D gene-RGD fusion construct can be selected. Combining protein extracts from a D gene minus lysogen with a D gene-RGD fusion lysogen will provide the full complement of necessary packaging proteins. Using this combination of lysogen extracts, bacteriophage genomes or minimal lambda cassettes can be encapsidated in vitro with a D gene-RGD fusion protein that can target the recombinant virus to cells expressing
25 RGD's cognate receptor. Preferably, the D-gene is modified to include in frame a ligand, e.g. cyclic RGD, so that the vector is internalized by the targeted cell type(s). This lysogen strain is incapable of producing virus because the COS sites are absent. The lysogen strains are grown to large quantities using standard bacterial culture techniques. Then, the lambda packaging proteins, including any modified forms of these proteins, can be obtained by standard methods,
30 such as freeze thawing and sonication of lysogen.

The packaging of the high capacity bacteriophage lambda vector is accomplished by mixing the purified engineered cosmid DNA with the isolated protein extract from the above

lysogen strain at approximately room temperature. This mixing results in the packaging of replication deficient modified bacteriophage lambda virus particles that contain both surface proteins as determined by the modified lysogen strain from which the packaging proteins are derived and a genome of an insert of up to about 50 kb flanked by COS sites yet lacking other bacteriophage coding sequences.

In the preferred embodiments, the bacteriophage genome further includes a regulatory sequence, e.g., a promoter region (also referred to as a "promoter"), that is operably coupled to the therapeutic polynucleotide. The regulatory sequence controls the expression of the therapeutic polynucleotide in the target cell. As used herein, a therapeutic polynucleotide (also referred to as "coding sequence") that encodes a therapeutic polynucleotide product, and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the transcription or the expression of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequence results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 3' or 5' non-transcribed and/or non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, CAAT sequence, and the like. Especially, such 5' non-transcribing regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream 5' or downstream 3' transcriptional regulatory sequences as desired. The bacteriophages of the invention optionally include 5' leader signal, or membrane integration sequences 5' or 3'.

Exemplary promoters that are useful for protecting the instant invention are provided in Table 5, which shows both constitutive promoters and regulatable promoters (e.g., cell lineage

specific promoters, inducible promoters). Exemplary constitutive promoters also are included in Table 5. In the preferred embodiments, the constitutive promoter is selected from the group consisting of a promoter of the phosphoglycerokinase gene, a long terminal repeat (LTR) of retroviruses, e.g., Rous sarcoma virus, Moloney murine leukemia virus. Exemplary tissue or cell specific transcriptional regulatory sequences are derived from the genes encoding the following proteins: tyrosinase, lipoprotein lipase, albumin, muscle creatine kinase, keratin (K14/K10), globin gene cluster, immunoglobulin heavy chain gene cluster, and involucrin. Several liver-specific promoters, such as the albumin promoter/enhancer, also have been described (see, e.g., PCT application number PCT/US95/11456, having international publication number WO96/09074, entitled "Use of a Non-mammalian DNA Virus to Express an Exogenous Gene in a Mammalian Cell," hereinafter WO 96/09074, and the references cited therein). In particular, the alpha-feto protein promoter, can be used to effect expression of a therapeutic polynucleotide(s) in liver tumor cells (but not normal liver cells) for treating liver cancer. Exemplary inducible promoters are identified in Table 5 and are described in the following references: Science 268:1786 (1995); TIBS 18:471 (1993); PNAS 91:3180 (1994); PNAS 90:1657 (1993); PNAS 88:698 (1991); Nature Biotechnol. 14:486 (1996); and PNAS 93:5185 (1996). The preferred inducible promoter system is the tetracycline inducible system. An exemplary repressible promoter, the tetracycline repressible system, is identified in Table 5 and is described in PNAS 89:5547 (1992).

A tetracycline inducible promoter system includes, for example, two tandem constructs: an appropriate promoter operably linked to a trans-activating polypeptide coding sequence (rtTA) (a mutated Tet R linked to a VP16) and poly A signal in tandem with a tetracycline responsive element (tetO and a eukaryotic minimal promoter) operably coupled to a coding sequence of interest. Containing a poly A site, these two tandem constructs can be, optionally, joined into a single construct separated by an internal ribosomal entry site (IRES). The tetO driven coding sequence is 5' to the mammalian promoter driven coding sequence. Addition of tetracycline, doxycycline, or derivatives thereof, activates the transactivating polypeptide to bind tetO and, in turn, to drive transcription of a polycistronic message including first the coding sequence of interest and the transactivator. The IRES allows initiation of translation of the trans-activating polypeptide or the coding sequence of interest, independently, from a single polycistronic message. In the absence of tetracycline, the transactivator is not activated and the tetO driven transcription of the coding sequence of interest is substantially reduced to negligible

levels.

Preferably, the bacteriophage genome further includes an enhancer region ("enhancer"). Exemplary enhancers that are useful for practicing the instant invention are provided in Table 5. The preferred enhances are selected from the group consisting of the following: a locus control region (beta-globin enhancer), an immunoglobulin gene enhancer, a cytomegalovirus (CMV) enhancer, a muscle creatine kinase enhancer, and an SV40 enhancer. Optionally, the bacteriophage genome can be engineered to contain an origin of replication to effect autonomous replication and facilitate persistence of the therapeutic polynucleotide in the mammalian cell. Origins of replication derived from mammalian target cells have been identified (see, e.g., Burhans, et al., 1994, Science 263: 639-640).

The bacteriophages optionally contain one or more sequences that are suitable for use in the identification of cells that have or have not been transfected. "Transfection", as used herein, refers to the introduction of the bacteriophage genome into the target cell. Markers to identify cells that have been transfected include, for example, genes encoding proteins that increase or decrease resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes having activities that are detectable by standard assays known in the art and genes which detectably (e.g. visibly) effect the phenotype of the transfected target cells, hosts, or plaques. Exemplary genes that are suitable as markers include the lacZ genes, chloramphenicol acetyltransferase genes, alkaline phosphatase genes, luciferase genes, and green fluorescent protein genes.

The bacteriophages of the invention contain on their surface a ligand that selectively binds to a receptor expressed on the surface of the target cell. The bacteriophage ligand binds to the target cell receptor to form a ligand-receptor complex that is internalized by the target cell. In this manner, the bacteriophage can be targeted for delivery to a pre-selected cell or tissue type, i.e., by selecting a ligand/receptor pair wherein the receptor is selectively expressed on a specific population of cells. Exemplary pairs of ligands/receptors which are useful in accordance with the methods of the invention are provided in Table 6.

The preferred ligand/receptor pairs for use in accordance with the methods of the invention include the following: insulin/insulin receptor, heregulin/heregulin receptor, keratinocyte growth factor/keratinocyte growth factor receptor, hepatic growth factor/hepatic growth factor receptor, RGD peptides/integrin alpha-5:beta-1, interleukin-2/interleukin-2 receptor, galactose/asialoglycoprotein, low density lipoprotein (LDL) or apoB100/LDL receptor,

very low density lipoprotein (VLDL), apoE/VLDL receptor, or HDL or apoA1/HDL receptor. In addition to these ligand/receptor pairs, antibodies can be attached to the bacteriophage via interaction of a bacteriophage coat protein specific antibody with its cognate antigen or via interaction of a recombinant bacteriophage coat protein that contains avidin and a biotinylated antibody. Once the antibody is tightly bound to the bacteriophage as described above, the antibody can direct the bacteriophage either to cells that express the Fc receptor in the case of a coat protein specific IgG antibody or to cells that express the cognate antigen of the attached biotinylated antibody. Selective ligand-receptor interaction also is useful for mediating internalization of the bacteriophage into subcellular locations e.g., the nucleus, mitochondria, and other membranes-bound organelles or cytoplasmic molecular aggregates of protein and/or nucleic acid.

In addition to the well-known ligand/receptor pairs for delivering a ligand-labeled component to a particular cell type, novel ligands can be identified using phage display procedures such as those described in (S. Hart, et al., J. Biol. Chem. 269(17):12468 (1994)). While such filamentous phages could, of course, never be used to deliver genetic material to a cell (because they are single stranded), this methodology is potentially very useful in the discovery of novel receptor ligand interactions. In general, phage display libraries using, e.g., M13 or fd phage are prepared using conventional procedures such as those described in the foregoing reference. The libraries display inserts containing from 4 to 80 amino acid residues. The inserts optionally represent a completely degenerate or a biased array of peptides. Ligands that bind selectively to a particularly type of target cell (e.g., mammalian cell) are obtained by selecting those phages which express on their surface a ligand that binds to the target cell of interest. These phages then are subjected to several cycles of reselection to identify the peptide ligand-expressing phages that have the most useful binding characteristics. Typically, phages that exhibit the best binding characteristics (e.g., highest affinity) are further characterized by nucleic acid analysis to identify the particular amino acid sequences of the peptides expressed on the phage surface and the optimum length of the expressed peptide to achieve optimum binding to the target mammalian cell. Alternatively, such peptide ligands can be selected from combinatorial libraries of peptides containing one or more amino acids. Such libraries can further be synthesized which contain non-peptide synthetic moieties which are less subject to enzymatic degradation compared to their naturally-occurring counterparts. These novel ligands can be attached to the bacteriophage surface to deliver the phage to the particular target cell of

interest. Alternatively, the ligands may be selected from polynucleotide libraries, as polynucleotides can also demonstrate specific affinity for polypeptide cell surface receptors. Exemplary screening methods for selecting bacteriophage vectors that transduce mammalian target cells via receptor-mediated endocytosis and target cells that internalize the bacteriophage vectors by this mechanism are described in the Examples.

At least four general procedures are available for attaching a ligand to the surface of a bacteriophage. These include (1) chemical modification of the bacteriophage surface (e.g., galactosylation, cross linking reactions); (2) modification of the bacteriophage genome to express a ligand on the bacteriophage surface (e.g., a fusion protein formed between the ligand and a functional viral packaging protein); (3) selective binding of a ligand (e.g., a monoclonal antibody, a polyclonal antibody, or functionally active fragments thereof containing an Fc domain) to a bacteriophage surface antigen to mediate targeting of the bacteriophage to cells that express an Fc receptor on their surface; and (4) modification of the bacteriophage genome to express a surface avidin-bacteriophage coat protein fusion product to which a biotinylated ligand (e.g., antibody) can be attached.

The simplest method for attaching a ligand to the surface of a bacteriophage is a chemical modification reaction in which the surface of the bacteriophage is subjected to galactosylation or lactosylation via N-linked glycosidic covalent linkages so as to attach galactose or lactose, respectively, to the bacteriophage surface. See, e.g., the Examples. Galactose- or lactose-labeled bacteriophages selectively bind to asialoglycoprotein receptors on the surface of hepatocytes to form a ligand-bacteriophage complex that is internalized by the target cell. Chemical modification also can be used to attach a peptide ligand to the bacteriophage surface. For example, peptide ligands containing a free amine group, carboxyl group, or sulfhydryl group can be attached to the bacteriophage surface using conventional procedures known to those of ordinary skill in the art for cross linking proteins. See, e.g., U.S. Patent No. 5,108,921, issued to Low et al. If the peptide ligand does not have a free amine or carboxyl group, such a group can be introduced by, for example, introducing a cysteine (containing a reactive thiol group) into the peptide ligand by site directed mutagenesis. Disulfide linkages can be formed between thiol groups in, for example, the peptide ligand and a protein expressed on the surface of the bacteriophage. For example, covalent linkages can be formed using bifunctional crosslinking agents that are known by those of ordinary skill in the art to have utility with respect to crosslinking peptides and proteins. Exemplary crosslinking agents include bismaleimidohexane

(which contains thiol-reactive maleimide groups and which forms covalent bonds with free thiols). See, also, the Pierce Co. Immunotechnology Catalogue and Handbook Vol. 1 for a list of exemplary homo- and hetero-bifunctional crosslinking agents, thiol-containing amines and other molecules with reactive groups for a comprehensive list of commercially available agents and corresponding peptide coupling chemistries that can be used to attach a peptide ligand to, for example, an amino acid functional group (e.g., amine) on the surface of a bacteriophage. Further exemplary coupling chemistries that are suitable for this purpose include methods which utilize the following crosslinking agents: glutaraldehyde (M. Riechlin, *Methods in Enzymology* 70:159-165 (1980); N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (T.L. Goodfriend, et al., *Science* 144:1344-1346 (1964); and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (M.H. Klapper and I.M. Klotz, *Methods in Enzymology* 25:531-536 (1972)). In general, the ligand-labeled bacteriophages can be prepared by using well-known methods for forming amide, ester or imino bonds between acid, aldehyde, hydroxy, amino, or hydrazo groups on the respective bacteriophage and ligand. In certain embodiments, reactive functional groups that are present in the amino acid side chains of the bacteriophage extracellular proteins or peptide ligands preferably are protected with a protecting group prior to coupling the ligand to the bacteriophage. As used herein, "protecting group" refers to a molecule which is bound to a functional group on a bacteriophage or peptide ligand and which may be selectively removed therefrom to expose the functional group in a reactive form. Preferably, the protecting groups are reversibly attached to the functional groups and can be removed therefrom using, for example, chemical or other cleavage methods. Thus, commercially available side-chain-blocked amino acids (e.g., Fmoc-derived amino acids from Advanced Chemtech, Inc., Louisville, KY) can be used to synthesize peptide ligands in accordance with standard peptide synthesis methods to obtain peptide ligands having side-chain-blocked amino acids. Alternatively, the peptide ligand side chains can be reacted with protecting groups after peptide ligand synthesis, but prior to the covalent coupling reaction. In this manner, the ligand-labeled bacteriophages of the invention can be prepared in which the amino acid side chains of the peptide ligand do not participate to any significant extent in the coupling of the peptide ligand to functional groups on the surface of the bacteriophage.

Alternatively, genetic engineering methods can be used to attach a ligand to the surface of a bacteriophage. For example, a sequence encoding the ligand ("ligand coding sequence") can be inserted, in frame, into the bacteriophage genome adjacent to or within a bacteriophage protein that is expressed on the bacteriophage surface to provide a fusion protein that contains

both functional ligand and a functional viral packaging protein. (See, e.g., the Examples.)

According to yet another embodiment, the ligand-labeled bacteriophage is formed by selectively binding an antibody or functionally active fragment thereof (i.e., an antibody fragment containing at least one antigen-binding site) to an antigen that is contained on the surface of the bacteriophage (e.g., a bacterial extracellular protein). The selectively bound antibodies mediate targeting of the bacteriophage to a target cell that contains on its surface an Fc receptor (e.g., a phagocyte or antigen presenting cell). Antibodies that are useful in accordance with this aspect of the invention are antibodies that exhibit a sufficiently high binding-affinity for a bacteriophage antigen to result in little or no dissociation of the antibody-antigen complex under physiological conditions. In general, such antibody binding to the surface of the bacteriophage is performed by contacting the bacteriophage with an antibody that selectively binds to an antigen expressed on the bacteriophage surface under the same conditions that are used for performing an immunoassay, e.g., an ELISA, RIA. Exemplary conditions are described in Current Protocols in Immunology, ed. Coligan, J.E., et al., National Institutes of Health, John Wiley and Sons, Inc. (1994).

In a particularly preferred embodiment, the ligand is attached to the surface of the bacteriophage by means of an avidin/biotin complex. As used herein, "avidin" or "avidin peptide" refers to an avidin molecule, a streptavidin molecule, or a fragment or variant thereof that binds to biotin with an affinity that is approximately the same (i.e., within 10%) or greater than the affinity with which streptavidin binds to biotin. According to this embodiment, the bacteriophage is modified to express on its surface avidin or a portion thereof that selectively binds to biotin with the requisite binding affinity. Modification of the bacteriophage to express avidin is most easily accomplished by inserting the nucleic acid encoding avidin or a functionally active portion thereof into the bacteriophage genome such that the avidin or avidin portion is expressed on the bacteriophage surface. For example, the avidin can be inserted, in frame, into the D gene of the lambda coat protein, using well-defined restriction sites in the lambda phage. (See, e.g., the Examples and figures.) In this manner, an avidin-expressing bacteriophage is produced which serves as an intermediate for attachment of a biotinylated ligand to the bacteriophage surface.

Alternatively, avidin or a functionally active portion thereof can be chemically coupled to the bacteriophage surface using standard cross-linking chemistries, such as those described above. The avidin-labeled bacteriophage permits non-covalent, yet high affinity, attachment of

pre-selected biotinylated ligands to the bacteriophage surface for receptor-mediated targeted delivery to the mammalian target cell. Exemplary ligands which can be biotinylated in accordance with standard procedures are provided in Table 6. Alternatively, the bacteriophage can be biotinylated and an avidin-labeled ligand can be used to form the ligand-labeled bacteriophages described herein.

The bacteriophages of the invention are contacted with the target cell under conditions to permit selective binding of the ligand on the surface of the bacteriophage to the receptor on the surface of the target cell and to allow the bacteriophage to enter the target cell. Conditions which permit the binding of a receptor to its cognate ligand are the physiological conditions (e.g., the particular pH, ionic strength, viscosity) at which the ligands and receptors are known to bind to one another *in vivo* and the conditions at which the ligands and receptors are known to bind to one another *in vitro*, such as in receptor assays for determining the presence of a ligand in, for example, a biological fluid. Such conditions are known to those of ordinary skill in the art of receptor-mediated processes, such as receptor-based binding assays and receptor-mediated delivery of therapeutic agents to preselected tissues *in situ*.

In general, the conditions that allow the target cell to live and transcribe the therapeutic polynucleotide are the same conditions that permit selective binding of the ligand to the receptor and that allow the bacteriophage to enter the target cell. Optionally, the conditions that allow the cell to transcribe the therapeutic polynucleotide further include the addition of an inducer (see, e.g., Table 5) that activates an inducible promoter to induce transcription and translation of the therapeutic polynucleotide. The optimum conditions for inducing the transcription and translation of a therapeutic polynucleotide that is under the control of a particular inducible promoter can be determined by one of ordinary skill in the art using no more than routine experimentation. In general, for *in vitro* gene therapy, conventional tissue culture conditions and methods are used to sustain the mammalian cell in culture. For example, the mammalian cell can be allowed to live on a substrate containing collagen, e.g., type I collagen, or a matrix containing laminin, such as described in PCT application number PCT/US95/11456, having international publication number WO96/09074, entitled "Use of a Non-mammalian DNA Virus to Express an Exogenous Gene in a Mammalian Cell," and the references cited therein.

As used herein, "contacting", in reference to the bacteriophage and the target cell, refers to bringing the bacteriophage into sufficiently close proximity to the target cell to permit the receptor on the target cell to selectively bind to the ligand on the bacteriophage. Such conditions

are well known to those of ordinary skill in the art and are exemplified by the procedure provided in the Examples. See also, e.g., U.S. patent No. 5,108,921, issued to Low et al. which reports the conditions for receptor-mediated delivery of "exogenous molecules" such as peptides, proteins and nucleic acids to animal cells and U.S. patent No. 5,166,320, issued to Wu et al., which reports the conditions for the receptor mediated delivery of a ligand-gene conjugate to a mammalian cell. For a further discussion of the conditions and mechanisms by which receptor mediated delivery can be used to deliver an exogenous molecule into a target cell, and in particular, into a mammalian cell, see, e.g., S. Michael, et al., J.Biol., Chem. 268(10):6866 (1993), "Binding-incompetent Adenovirus Facilitates Molecular Conjugate-mediated Gene Transfer by the Receptor-mediated Endocytosis Pathway"; M. Barry, et al., Nature Medicine 2(3):299 (1996), "Toward cell-targeting gene therapy vectors: Selection of cell-binding peptides from random peptide-presenting phage libraries"; S. I. Michael, Gene Ther. 2:660 (1995), "Addition of a short peptide ligand to the adenovirus fiber protein".

The bacteriophage can be contacted with the targeted mammalian cell *in vitro*, for example, for *ex vivo* gene therapy or production of a catalytic RNA or recombinant protein in cell culture, or *in vivo* for *in vivo* gene therapy or *in vivo* production of a polynucleotide transcription or translation product. As used herein, a "mammalian target cell" refers to a mammalian cell (preferably, a human cell) which contains on its surface a receptor for the ligand that is contained (e.g., expressed) on the surface of the bacteriophage. Essentially, any mammalian cell can be targeted in accordance with the methods of the invention. The cell may be a primary cell or may be a cell of an established cell line. Exemplary cell types that can be targeted in accordance with the methods of the invention are provided in Table 4. Preferably, the mammalian cell is a hepatocyte (liver cell), a breast epithelial cell, a keratinocyte, a melanocyte, or a hematopoietic cell, e.g., erythrocyte, leukocyte, monocyte, or a lymphocyte. Screening methods, such as those described in the Examples, can be used to confirm that these and other target cells internalize the bacteriophage vectors of the invention via receptor-mediated endocytosis and, further, that these target cells express detectable levels of the exogenous polynucleotide insert. Such high-throughput screening methods can be used to select target cells that satisfy the above-noted criteria using no more than routine experimentation. In addition, such screening assays are predictive of receptor-mediated endocytosis of target cells *in vivo*.

Where the bacteriophage is contacted with the cell *in vitro*, the target cell subsequently can be introduced into the mammal (e.g., into the portal vein or into the spleen) if desired.

Accordingly, expression of the therapeutic polynucleotide is accomplished by allowing the cell to live or propagate *in vitro*, *in vivo*, or *in vitro* and *in vivo*, sequentially. Similarly, where the invention is used to express a therapeutic polynucleotide in more than one cell, a combination of *in vitro* and *in vivo* methods are used to introduce the therapeutic polynucleotide into more than one mammalian cell.

In *ex vivo* gene therapy, the cells are removed from a subject and a therapeutic polynucleotide is introduced (i.e., transfected) into the cells *in vitro*. Typically, the transfected cells are expanded in culture before being reimplanted into the mammalian recipient. The procedure for performing *ex vivo* gene therapy is outlined in U.S. Patent 5,399,346 and in exhibits submitted in the file history of that patent, all of which are publicly available documents. In general, *ex vivo* gene therapy involves the introduction *in vitro* of a functional copy of a gene into a cell(s) of a subject which contains a defective copy of the gene, and returning the genetically engineered cell(s) to the subject. The functional copy of the gene is under the operable control of regulatory elements which permit expression of the gene in the genetically engineered cell(s). In *in vivo* gene therapy, the target cells are not removed from the patient. Rather, the therapeutic polynucleotide is introduced into the cells of the mammalian recipient *in situ*, i.e., within the recipient. In general, the improved gene therapy method disclosed herein is practiced by using the ligand-labeled bacteriophage of the invention in place of the gene therapy vectors disclosed in the prior art (e.g., adenoviral vectors, modified adenoviral vectors, retroviral vectors, plasmids, liposomes) in the procedures currently used for administering these vectors (or cells containing these vectors) to the subjects. Such procedures are known to those of skill in the art of human gene therapy. (See, also, the references identified below with respect to *in vivo* gene therapy.)

A particularly preferred embodiment of the invention is illustrated in the Examples, which describes the construction of a lambda bacteriophage for use as a gene therapy vector. Briefly, a bacteriophage containing an exogenous polynucleotide (encoding beta-galactosidase) was chemically modified (galactosylated) to contain galactose residues on the bacteriophage surface. The galactose-labeled bacteriophage was internalized by HepG2 cells (a hepatoma cell line) in culture, presumably, by binding of the labeled bacteriophage to the asialoglycoprotein receptor present on the surface of the hepatocytes.

Insertion of the therapeutic polynucleotide into the target cell genome may be either transient or permanent. By "transient", it is meant that the bacteriophage genome lacks the

capacity to replicate and/or to segregate to progeny cells. For example, the therapeutic polynucleotide may be epigenetic and without the capacity to replicate and segregate to progeny cells (e.g., lacks an origin of replication, appropriate telomere and centromere structures). "Transient" insertion into the target cell also occurs, for example, when the bacteriophage is used to infect cells of limited replicative capacity, i.e., non-stem cells. "Permanent" insertion of the therapeutic polynucleotide into the target cell is accomplished by, for example, (1) infecting stem cells that produce bacteriophage-bearing progeny; or (2) including recombination sequences in the bacteriophage genome on either side of the therapeutic polynucleotide so as to promote reasonably efficient homologous recombination of the therapeutic polynucleotide into a defined sequence of the target cell genome or (3) random integration into the host cell chromosomal DNA. "Permanent" insertion also can be achieved by including in the bacteriophage an origin of replication, telomeres and centromeres to obtain a bacteriophage that autonomously replicates (i.e., an "artificial chromosome") and is capable of segregating into progeny cells. If the bacteriophage genome is autonomously replicating, it is preferred to further include in the bacteriophage genome appropriate enhancer-promoter sequences, such as those described in the aforementioned tables.

For *in vivo* gene therapy, the bacteriophage is administered to the mammalian recipient, for example, intravascularly, intraluminally (introduction of the bacteriophage into body cavities and lumens, such as the genital urinary tract, gastrointestinal tract, trachea-bronchopulmonary tree or other internal tubular structures), direct injection into a tissue (e.g., muscle, liver), topical application (e.g., eye drops or aerosol application to mucosal surfaces), or intracavitary (e.g., intraperitoneally or intrathecally (introduction into the cerebrospinal fluid). Optionally, an implantable pump or other device or implant (preferably, a bioerodible implant) to effect the sustained release of the bacteriophage can be used to facilitate delivery of the bacteriophage to the mammalian targeted cell over a pre-selected period of time (e.g., sustained release over a period of days to sustained release over a period of weeks to months). Although the ligand/receptor-mediated delivery of the bacteriophage is the predominant mechanism for targeting delivery of the bacteriophage to a particular cell type, delivery to the target cell can further be modulated by regulating the amount of bacteriophage administered to the mammalian recipient and/or by controlling the method of delivery. Thus, for example, intravascular administration of the bacteriophage to the portal vein or to the hepatic artery can be used to facilitate targeting the bacteriophage to a liver cell.

In general, the bacteriophage can be administered to the mammalian recipient using the same modes of administration that currently are used for adenovirus-mediated gene therapy in humans. Such conditions are adequate for contacting the bacteriophage and the target cell under conditions to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and to allow the bacteriophage to enter the target cell. These conditions are described in the following references: PNAS 90:10613 (1993); Nature Medicine 1:1148 (1995); Nature Medicine 12:266 (1996); New Engl. J. Med. 333:832 (1995); and New Engl. J. Med. 333:823 (1995). Preferably, the bacteriophage is administered to the mammalian recipient by intravascular injection, intra-organ introduction by, for example, injection into the organ or contacting the bacteriophage with the organ in the presence of a tissue permeabilizing agent; and introduction of the bacteriophage into body cavities or lumens. Optionally, immunosuppressive drugs, such as glucocorticosteroids or cyclophosphamide are co-administered with the bacteriophage to suppress a primary immune response that may be triggered by an initial exposure to a foreign antigen. Mammalian cells which have been transfected with the bacteriophage *ex vivo* can be introduced into the mammalian recipient using the known methods for implanting transfected cells into a human for gene therapy. See, e.g., U.S. Patent No. 5,399,346 ("Gene Therapy") issued to Anderson et al.; PCT International application no. PCT/US92/01890 (Publication No. WO 92/15676, "Somatic Cell Gene Therapy", claiming priority to U.S. Serial No. 667,169, filed March 8, 1991, inventor I. M. Verma); PCT International application no. PCT/US89/05575 (Publication No. WO 90/06997, "Genetically Engineered Endothelial Cells and Use Thereof", claiming priority to U.S. Serial No. 283,586, filed December 8, 1989, inventors Anderson, W.F. et al.).

The invention is not limited in utility to human gene therapy, but also can be used in the manufacture of a wide variety of proteins and nucleic acids that are useful in the fields of biology and medicine. The bacteriophages of the invention advantageously provide a method for synthesizing gene products from genes which range in size from about one to one-hundred kilobases. Further, the invention provides a method for providing the bacteriophage vectors at a low cost, namely, by propagating the bacteriophages in a prokaryotic host. Moreover, the invention provides a simple method for preparing mammalian proteins, including proper post-translational modifications, *in vitro*. Thus, the invention provides an improved method for introducing an exogenous polynucleotide into a mammalian cell. The improved method involves contacting the bacteriophages of the invention (which contain the exogenous polynucleotide).

with the mammalian cell and allowing the bacteriophage to enter the cell, gain access to the nucleus, and replicate the exogenous polynucleotide therein. As discussed above, the bacteriophage contains on its surface the ligand that selectively binds to a receptor for the ligand that is contained on the surface of the mammalian cell.

As used herein, an "exogenous polynucleotide" refers to a nucleic acid that is not normally present in the bacteriophage genome and that is inserted into the bacteriophage using recombinant engineering methodology. Examples of exogenous polynucleotides include the above described therapeutic polynucleotides, as well as regulatory polynucleotides which are not intended for therapeutic applications (e.g., polynucleotides that are introduced into the mammalian cell *in vitro* or *in vivo* for the purpose of producing a mammalian protein/protein complex *in vitro* or *in vivo*).

According to yet another aspect of the invention, an avidin-labeled bacteriophage that is used as an intermediate in connection with the above described methods for introducing a therapeutic or other exogenous polynucleotide into a mammalian cell is provided. The intermediate can be used to prepare a bacteriophage having on its surface virtually any ligand, provided, that the ligand can be biotinylated and retain its binding activity to a receptor. Thus, the invention also provides a method for preparing a ligand-labeled bacteriophage which involves contacting an avidin-expressing bacteriophage with a biotinylated ligand under conditions to permit binding of the avidin to the biotin. As will be apparent to those of ordinary skill in the art, alternative binding pairs can be used in place of the avidin-ligand binding pairs to accomplish this same objective. Such binding pairs include, for example, streptavidin-biotin binding pairs, antibody antigen, and any other high affinity interactions. Thus, the invention provides a generic mechanism for forming a bacteriophage which contains on its surface virtually any type of ligand. The avidin-labeled bacteriophage can be provided as a component of a kit for labeling a bacteriophage with a ligand of choice. The kit includes instructions for forming a ligand-labeled bacteriophage by allowing the avidin-bacteriophage to react with a biotinylated ligand under conditions to permit selective binding of the avidin-labeled bacteriophage to the biotinylated ligand. Optionally, the kit further includes reagents, and appropriate instructions, for biotinylating a ligand of choice.

The invention provides other compositions and kits which are useful for practicing the above-described methods. According to a particularly preferred aspect of the invention, a bacteriophage of the invention is provided. The bacteriophage contains (a) a bacteriophage

genome containing an exogenous polynucleotide that can be transcribed in a mammalian cell; and (b) a ligand contained on the surface of the bacteriophage that selectively binds to a receptor expressed on the surface of a mammalian cell. The bacteriophages of the invention optionally are contained in a pharmaceutically acceptable carrier to form a pharmaceutical composition.

5 The pharmaceutical compositions should be sterile and contain a therapeutically effective amount of the bacteriophages (or target cells containing the bacteriophages) in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is
10 compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

In one particular embodiment, the preferred pharmaceutical composition is contained in
15 an implant that is suitable for implantation into the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. PCT/US/03307 (Publication No. WO 95/24929, entitled "Polymeric Gene Delivery System", claiming priority to U.S. patent application serial no. 213,668, filed March 15, 1994). PCT/US/03307 describes a biocompatible, preferably biodegradable polymeric matrix for
20 containing an exogenous gene under the control of an appropriate promoter. The polymeric matrix is used to achieve sustained release of the exogenous gene in the patient. In accordance with the instant invention, the bacteriophage particles described herein are encapsulated or dispersed within the biocompatible, preferably biodegradable polymeric matrix disclosed in PCT/US/03307. The polymeric matrix preferably is in the form of a micro particle such as a
25 micro sphere (wherein the bacteriophage particle is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein the bacteriophage particle is stored in the core of a polymeric shell). Other forms of the polymeric matrix for containing the bacteriophage particle include films, coatings, gels, implants, and stents. The size and composition of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix device is implanted. The size of the polymeric matrix further is selected according to the method of
30 delivery which is to be used, typically injection into a tissue or administration of a suspension by aerosol into the nasal or pulmonary areas. The polymeric matrix composition can be

selected to have both favorable degradation rates and also to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when the polymeric matrix is administered to a mucosal surface. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver the bacteriophage particles of the invention to the subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. Synthetic polymers are preferred. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multi-valent ions or other polymers.

In general, the bacteriophage particles of the invention are delivered using the bioerodible implant by way of diffusion, or more preferably, by degradation of the polymeric matrix.

Exemplary synthetic polymers which can be used to form the biodegradable delivery system include: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terphthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, poly-vinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terphthalate), poly(vinyl alcohols), polyvinyl acetate, polyvinyl chloride, polystyrene and polyvinylpyrrolidone.

Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof.

Examples of biodegradable polymers include synthetic polymers such as polymers of

lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(butic acid), poly(valeric acid), and poly(lactide-cocaprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water *in vivo*, by surface or bulk erosion.

Bioadhesive polymers of particular interest include bioerodible hydrogels described by H.S. Sawhney, C.P. Pathak and J.A. Hubell in Macromolecules, 1993, 26, 581-587, the teachings of which are incorporated herein, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate). Thus, the invention provides a composition of the above-described bacteriophages for use as a medicament, methods for preparing the medicament and methods for the sustained release of the medicament *in vivo*. In the preferred embodiments, the bacteriophage is selected from the group of bacteriophages shown in Table 1 and is labeled with an appropriate ligand as described above. Preferably, the bacteriophage is selected from the group consisting of a lambda phage, a P1 phage, a P22 phage, and an SPP1 phage; more preferably, the bacteriophage is a lambda phage or a P1 phage; most preferably, the bacteriophage is a lambda phage.

Examples

Introduction to the Examples

In the present method, a bacteriophage particle serves as a vector for gene delivery to a mammalian cell. The bacteriophage capsid and/or tail are modified to contain a ligand (or an adapter, e.g., avidin, for a ligand) that can bind to a receptor contained on the surface of a mammalian cell and facilitate entry therein. Following receptor-ligand interaction the recombinant bacteriophage can be internalized via receptor mediated endocytosis or other receptor-mediated mechanisms of intracellular transport. The bacteriophage genome is engineered to include transcriptional control signals, which allow expression of an exogenous polynucleotide in the mammalian cell. Optionally, the recombinant bacteriophage further contains an endosomal lysis signal, such as that contained within the capsid-stabilizing protein VI and L3/p23 of adenovirus (Greber UF et al. EMBO J, 1996; 15: 1766) to promote export of the bacteriophage genome into the cytoplasm prior to fusion of the endocytosed vesicles with mammalian cell lysosomes and enzymatic degradation of the bacteriophage genome. Conventional recombinant DNA techniques can be used for inserting these and other nucleic acid sequences into the phage genome. For example, the adenovirus endosomal lysis signal can be inserted in-frame into or appended to the bacteriophage tail fiber J gene.

To effect successful transduction after entry of the recombinant bacteriophage into the cytoplasm, the bacteriophage genome is delivered to the nucleus. In dividing cells, the genome of the bacteriophage used in the invention is efficiently delivered to the nucleus during mitosis when the nuclear membrane dissipates and reforms. For delivery of the bacteriophage genome to the nucleus of non-dividing cells, the phage particle is modified to include nuclear localization signals (NLS) that mediate transport of molecules or macromolecular aggregates, such as viral DNA across the nuclear membrane and into the nucleoplasm. Such nuclear localization signals are known to those of ordinary skill in the art and include portions of the human immunodeficiency virus (HIV) proteins matrix (MA) and Vpr (Naldini L et al. Science 1996; 272: 263; Bukrinsky MI et al., Nature 1993; 365: 666), large T antigen of SV40 (Kalderon D et al. Cell 1984; 39: 499; *Drosophila* Antennapedia (Derossi D et al. J Biol Chem 1996; 271: 18188), VP22 of Herpes virus (Elliott G. et al., Cell 1997; 88: 223), *X. laevis* nucleoplasmin, *X. laevis* lamin L, human c-myc encoded protein, and adenovirus type 2/5 Ela (Chelsky D et al. Mol

Cell Biol 1989; 9: 2487). For example, the HIV or SV40 nuclear localization signal can be inserted in frame into or appended to the tail fiber J protein or the H protein which is thought to bind tightly to the bacteriophage genome. Exemplary nuclear localization signals as disclosed in the above-identified references are provided in SEQ. ID NOS. 7-14.

5 In a preferred embodiment, the bacteriophage genome further includes adeno-associated viral sequences that mediate integration into specific chromosomal regions of the mammalian cell to effect delivery of the transduced bacteriophage genome to a specific mammalian cell chromosomal location. Such adeno-associated viral sequences are known to those of ordinary skill in the art. In yet other embodiments, the bacteriophage has a genome of sufficient size (e.g.,
10 P1 phage) to permit the further inclusion of nucleic acid sequences located 5' and 3' of the exogenous polynucleotide to promote site directed homologous recombination. More preferably, the bacteriophage has a genome of sufficient size (e.g., P1 phage) to permit insertion of a large genetic unit containing not only one or more coding sequences of interest and their associated control regions, but in addition, mammalian autonomous replicating sequences, as well as
15 centromere and telomere sequences in a self replicating format to form, in effect, an artificial chromosome. The ability to deliver exogenous genetic material of large size (e.g., a complete gene, including regulatory sequences) has obvious advantages with respect to achieving stable and tissue specific expression *in vivo* and *in vitro*.

A description of several bacteriophage vectors that can be used in accordance with the
20 methods of the invention follows. Briefly, Example I illustrates the construction of three bacteriophage λ vectors containing exogenous genetic material; Example II illustrates the post-packaging modification of bacteriophage λ capsid and tail proteins to form a galactose-labeled bacteriophage λ ; Example III illustrates the targeted delivery of a modified bacteriophage of the invention to human hepatoma G2 (HEP G2) cells grown *in vitro*; Example IV illustrates the
25 targeted delivery of a modified bacteriophage of the invention to hepatocytes in the liver of live mice *in vivo*; Example V illustrates the construction of bacteriophage vectors containing modified tail proteins; and Example VI illustrates screening methods for selecting bacteriophages and target cells that transduce mammalian cells via receptor-mediated endocytosis. These examples are provided for illustrative purposes only, and their inclusion is not meant to limit the
30 scope of invention. Thus, each of the procedures described in reference to the bacteriophage λ can be performed using any of the bacteriophages identified in Table 1 by: (1) obtaining the

published restriction maps for these bacteriophages; (2) selecting an exogenous polynucleotide (e.g., a gene having a published sequence that encodes a therapeutic polynucleotide product, such as identified in Tables 2 or 3); (3) inserting the gene into the well-defined restriction sites of the bacteriophage genome using substantially the same procedures described herein (e.g., blunt-ended ligation into bacteriophage lambda) or alternative procedures known to one skilled in the art for the insertion of a polynucleotide into a restriction site of a bacteriophage; (4) modifying the surface of the bacteriophage to include a ligand that targets delivery of the modified bacteriophage to a mammalian cell that contains on its surface the cognate receptor for the ligand (such as identified in Table 6) using substantially the same procedures described herein (e.g., galactosylation of the bacteriophage lambda phage particles, otherwise chemically modifying the surface of the bacteriophage to attach a ligand, or engineering the bacteriophage to express the ligand as a surface protein or polypeptide); and (5) contacting the modified bacteriophage with the target cell under conditions that permit the selective binding of the bacteriophage ligand to the cognate receptor using substantially the same conditions described herein for *ex vivo* and *in vivo* targeted delivery of bacteriophage lambda or alternative procedures known to one of ordinary skill in the art. By "substantially the same conditions", it is meant that the conditions are modified to substitute a different bacteriophage for bacteriophage lambda in the procedure and to use the published restriction enzymes, buffers, incubation times, size inserts and so forth that are known to be appropriate for modifying the different bacteriophage to include an exogenous polynucleotide and contain a surface ligand.

Example I Construction of three bacteriophage λ vectors

(a) Construction of the CMV- β -gal - lambda DASH II Chimeric DNA Sequences

The CMV β -gal I chimeric DNA sequences used in these experiments was constructed as follows. A 10.7 kilobase pair (kb) Eco RI fragment excised from plasmid adCMV/ NLS β -gal (obtained from Dr. Ronald Crystal, Columbia University; see Figure 3) that contained the reporter gene expression cassette was ligated to Eco RI/ BamHI double-digested left and right arm of λ DASH II (Stratagene, La Jolla, CA), a derivative of λ 2001, which contains an extended range of cloning sites. The genetic and physical map information used in this invention was obtained from published wild type λ DNA sequences and from the GenBank data base (accession number J02459).

The 10.7 kb reporter gene expression cassette included DNA sequences corresponding to

the CMV promoter/ enhancer linked to the E. coli β -gal gene. The expression cassette also included sequences encoding simian virus 40 (SV40) RNA splicing donor / acceptor sites and polyadenylation signals.

5 Generation of recombinant λ phage particles. CMV β -gal containing recombinant λ phage particles were generated by packaging the ligated CMV β -gal λ DASH II chimeric DNA with an *in vitro* packaging extract, Giga pak Gold II (Stratagene, La Jolla, CA), according to the manufacturer instructions.

10 Large scale preparation of recombinant phage particles. Conventional methods can be used to propagate the recombinant λ phage (e.g., Meth. Enzymol. 152:145-170, 1987). For example, the bacteriophage λ that was used in this experiment was grown and amplified from a single agar plug. Liquid lysates were made by growing the E. coli strain XL1 blue MR (P2) (genotype Δ (mcr A)) 183 Δ (mcr CB- hst MR) 173 end A 1 sup E 44 thi-1 gyr 96 rel A1 lac (P2 lysogen), to
15 A550 greater than or equal to 0.5 in Luria broth containing 5mM CaCl_2 at 37°C with vigorous shaking. At this point, the phages were added to a "moi" (multiplicity of infection) of 0.01. After 4-5 hrs of vigorous shaking, the lysed culture broth was centrifuged to remove cell debris. Amplified bacteriophages were concentrated by polyethylene glycol (PEG) precipitation from the lysate in accordance with standard procedures. PEG precipitated phages were further
20 purified by CsCl_2 gradient centrifugation in accordance with standard procedures. (See, e.g., Meth. Enzymol. 152:145-170, 1987 for standard PEG precipitation and CsCl_2 gradient purification protocols.)

25 Construction of phosphoglycerokinase (PGK) β -gal λ DASH II chimeric DNA sequences.

Expression from the CMV promoter/enhancer by mouse liver *in vivo* reportedly is extremely low (Furth, A., et al., Nucleic Acid Research 19:6205-08 (1991)). Accordingly, for *in vivo* targeted delivery of reporter β -gal gene to mouse liver, a chimeric PGK (phosphoglycerokinase) promoter/enhancer/ β -gal/ λ DASH II (Fig. 4) was constructed as follows. Eco RI and Bal I digested 0.6 kb fragment released from the PGK promoter containing plasmid (Gene, 80:65, 1987) was subcloned into Eco RI Xho I/ blunt site of adaCMV/ NLS/ beta-gal plasmid. The
30 resulting plasmid (designated PGK-NLS- β -gal) was Eco RI digested and ligated to λ DASH II

sequences. After ligation, the chimeric PGK-NLS- β -gal λ DASH II DNA was packaged *in vitro* and subsequently propagated in *E. coli* for large scale production of phage particles.

Construction of MCK-DMD- λ DASH II chimeric DNA sequence. As discussed previously, a significant advantage of the bacteriophage λ used in this invention is that it can be readily engineered to permit large gene-containing expression cassettes that could never be achieved by viral vectors currently used for delivery to mammalian cells. An example is a phage vector containing the MCK-DMD gene, which at 20.3 kb (Fig 5) greatly exceeds the capacity of currently used vectors. This vector was constructed as follows. The 20.3 kb expression cassette containing muscle creatine kinase (MCK) promoter/enhancer 5' to the full length DMD cDNA (obtained from J. Chamberlain, U. Michigan) was released from the plasmid pMDA after digestion with BssHII enzyme in accordance with standard procedures. This fragment was blunt ended and ligated to λ DASH II DNA sequences. The MCK-DMD gene / λ DASH II DNA sequences were used for generating the recombinant bacteriophage λ particles after packaging the chimeric DNA sequences with *in vitro* packaging extract (as described above).

Construction of λ phage particles displaying the ligand "L" heregulin for targeted gene delivery
Construction of λ phage particle that displays a specific ligand can be used for delivering the gene to a specific cell or tissue expressing the corresponding receptor via receptor/ligand mediated endocytosis. DNA sequences that encode ligand(s) known to be endocytosed after interaction with cognate receptor(s) were inserted, in frame, into the D-gene locus of the λ phage genome to produce a transcription template for a chimeric D gene-ligand bifunctional protein that expresses the fusion protein (including the ligand(s) of choice) on the bacteriophage surface. A similar strategy recently has been reported for targeting retrovirus vectors for gene delivery into a specific cell or tissue type (Proc. Natl. Acad. Sci. 92:9747-51 (1995)). For example, ligand directed retroviral targeting of human breast cancer cells recently has been reported by constructing retrovirus vectors expressing heregulin -gp70 chimeric envelope genes.

Alternatively, ligand directed λ phage targeting of specific mammalian cells is achieved by expressing chimeric λ D-gene and immunoglobulin single chain variable fragments (SCVF) directed against receptors such as LDL and IL-2, both of which are known to be internalized after the antibody-receptor interaction. Recently this approach has been used to generate

retrovirus vectors expressing chimeric envelope and SCVF for LDL receptor protein to transfer a β -gal gene to human cells expressing the LDL receptor (Proc. Natl. Acad. Sci. 92:7570).

Bacteriophage λ particles having a capsid that displays a chimeric λ D-gene-avidin fusion protein also can be used to effect targeted delivery of the phage particles to a specific mammalian cell. Since the avidin molecule has very high affinity for biotin, any peptide or polypeptide ligand that can be biotinylated without adversely affecting the ability of the receptor to mediate endocytosis can be used as described herein. The avidin gene, or portion thereof encoding the polypeptide that selectively binds to biotin, is inserted, in frame, into the bacteriophage genome using the procedures described herein for forming a chimeric λ D-gene and the published cDNA nucleic acid sequence for avidin (Gope, L. Mohan, et al., Nucleic Acid Research 15:3595-3606 (1987) and GenBank Accession No. 451889).

Fig. 6 illustrates the construction of a λ phage particle expressing the heregulin-D-gene chimeric DNA sequences on its capsid. In this construction, the D gene heregulin sequences were first fused by generating 3 fragments (AB, CD, and EF) by polymerase chain reaction (PCR) in accordance with standard procedures using Taq polymerase and 6 sets of primers (a,b,c,d,e,f):

primer a (SEQ. ID NO. 1) ATACCGAGGGCTGCAGTGTACA

primer b (SEQ. ID NO. 2) CTCTTTCAATTGGGGAGGCAAAACGATGCTGATTGCCGTTT

primer c (SEQ. ID NO. 3) TTGCCTCCCCAATTGAAAGAG

primer d (SEQ. ID NO. 4)

GTGATGAAGGGTAAAGTTATTTGCGTTTTTTTTTCGGCGGGGTCCTCCATAAATT
CAATC

primer e (SEQ. ID NO. 5)

TAACTTTACCCTTCATCACTAAAGGCC

primer f (SEQ. ID NO. 6)

AAACGTACAGCGCCATGTTTACCAG

Gel purified fragments AB, CD and EF were treated with T4 polymerase to remove the overhanging nucleotide "A" in the fragment generated by terminal transferase activity present in the Taq polymerase used for PCR amplification. This step ensures the joining "in frame" of the coding sequences of both the λ D-gene and heregulin. Next,

fragments AB and CD were joined by first denaturing and then annealing the partial overlapping sequences, followed by extension with Taq polymerase, and then amplification after the addition of primers a and d. The fragment AD was gel purified, treated with T4 polymerase, and subsequently used for joining fragment EF (as described above). The fused λ D-gene/hereregulin gene containing fragment AF was subcloned into the TA cloning vector (Invitrogen, San Diego, CA). The clones containing the AF fragment were identified by determining the sequences with a double-stranded sequencing method using the Sequenase 2.0 kit (USB, Cleveland, OH). The AF fragment was generated after digestion of the TA plasmid clone with the restriction enzyme BsrGI and ligated into the necessary fragments of λ (as shown in Fig. 6). This chimeric DNA construct was used to generate λ phage particles having a genome that includes the recombinant D-gene/hereregulin construct and a capsid that displays this chimeric protein.

Example II

Post-packaging modification of bacteriophage λ capsid and tail proteins to form a galactose-labeled bacteriophage λ

Chemical Modification.

The particular procedure for modifying the bacteriophage λ capsid and tail proteins to form a galactose-labeled bacteriophage λ is described herein. The galactose-labeled bacteriophage particles can be used to target liver cells which express a unique asialo-glycoprotein receptor. This procedure is based upon the published procedures for forming an artificial asialo-glycoprotein containing lactose (Neda, H., et al., JBC 296:14143-14146 (1991)) or galactose (Human Gene Therapy 5:429-435 (1994)).

Method. CsCl₂ purified CMV β -gal reporter gene containing λ phage particles (10^{11} PFU/ml) were galactosylated in 2 ml reaction volume containing 60mg of galactose and 100 mg of 1 ethyl-3-(3-diethylaminopropyl) carbodiiminide (EDC, Sigma Chemical Corp., Saint Louis, MO) in sodium chloride solution. The pH of the unbuffered solution was adjusted to 7.5 with NaOH and the reaction mixture was incubated at room temperature for various lengths of time ranging from 24 to 48hrs. The galactose associated with phage particles was determined using ¹⁴[C]-labeled galactose. Twenty five μ l of ¹⁴[C]-galactose (250-360mCi/mM, DuPont NEN, Boston, MA) were included

in a 100:1 reaction mixture except the cold galactose was omitted. At the end of the different incubation periods, the solutions were filtered and washed through nitrocellulose filters in a vacuum filtration device. The filters were removed and counted in a Beckman scintillation counter (Palo Alto, CA). The radioactivity on filters was converted to numbers of ^{14}C -galactose on the basis of the specific activity. The extent of galactosylation was expressed as numbers of galactose/phage particles.

As described in the detailed description of the invention, a variety of chemical methods can be used to attach a ligand to bacteriophage capsid and/or tail proteins. For example, small peptide ligands, such as insulin, epidermal growth factor (EGF), keratinocyte growth factor (KGF), Fab fragments for anti-polymeric immunoglobulin receptors can be covalently linked to λ phage using the hetero-bifunctional crosslinking reagent N-succinimidyl 3-(2-pyridyl dithio) propionate (SPDP) or other bifunctional crosslinking agents in accordance with manufacturer's instructions. Additional crosslinking agents are provided in the description and are known to those of ordinary skill in the art. After coupling the ligand to the bacteriophage surface proteins, the modified bacteriophage is used to selectively deliver the gene of interest to target cells that express a cognate receptor for the ligand (Example III).

Example III

Targeted delivery of a Modified Bacteriophage of the Invention to human hepatoma G2 (HEP G2) cells grown *in vitro*

In vitro targeted delivery of CMV β -gal expression cassette containing galactose modified λ phage particles.

1. Growth of cells:

Conventional tissue culture methods were used to grow HEP G2 cells. HEP G2 cells were cultured in minimal essential medium as modified by Eagle (EMEM) containing 10% FBS. Cells were seeded one day prior to the addition of λ phage particles for gene transfer experiments. *In vitro* targeted delivery of the β -gal gene to HEP G2 cells was accomplished by allowing the phage particles to interact with the cells in tissue culture growth medium for about 6-10 hrs; more preferably, for 8-10hrs. In general, after galactosylation, 10^7 - 10^{10} total phage particles/ml for 6-10 hrs, preferably 10^7 particles/ml for 10 hours, are needed for efficient transduction of targeted cells. After

exposing the cells to phage particles for the appropriate time, the phage-containing medium was removed and replaced with fresh media.

2. Detection of internalization of bacteriophage vectors and gene expression:

After ligand-receptor mediated endocytosis of a bacteriophage vector into a mammalian cell, the expression of the exogenous genetic material in the mammalian cell can be monitored using standard methodologies. For example, delivery of a bacteriophage modified with a CMV β -gal recombinant construct and a galactose ligand to a HEP G2 cell receptor can be measured by detecting bacteriophage DNA or RNA by Southern or northern blotting or *in situ* hybridization with or without amplification by polymerase chain reaction (PCR). Radioactively-labeled DNA or RNA probes that selectively hybridize to unique portions of the phage genome, e.g., portions corresponding to a regulatory sequence, such as a promoter, or portions corresponding to the exogenous genetic material, such as the reporter gene β -galactosidase, can be designed and constructed using standard molecular biology techniques. Where the modified bacteriophage is to be used for delivering exogenous genetic material to a mammalian cell *in vivo* (e.g., to hepatocytes in murine liver), delivery of the phage to the specific cell can be detected by obtaining the targeted cells in a biopsy and assaying the biopsied cells using, for example, the above-mentioned conventional methods (e.g., Southern or northern blotting).

In general, expression of a gene of interest (e.g., therapeutic or marker polynucleotide) in a mammalian cell is detected by measuring the functional or immunological activity of the expressed gene in the targeted tissue, targeted cell, or body fluid (e.g., serum, lymph fluid). Alternatively, direct RNA or protein analysis for detecting specific transcription or translation products can be performed in accordance with standard practice. Exemplary detection techniques to measure gene expression include one or more of the following techniques, alone or in combination: northern or western blotting, *in situ* hybridization, reverse transcription, PCR amplification, immunostaining, RIA and ELISA. Such routine techniques also can be used to measure the stability of expression and maintenance of the delivered exogenous genetic material in the mammalian target cell by, for example, measuring the expression of one or more

reporter (e.g., marker) genes as a function of time following transduction. In an exemplary protocol, a reporter gene #1 initially is delivered to the target mammalian cells. One week later, reporter gene #1 expression is assayed. One to three months following the initial transduction, a reporter gene #2 is transduced into the same population of target cells. One week later, the ratio of reporter gene #1 to reporter gene #2 is determined by, for example, immunostaining (e.g., using a different dye for each immunohistologic assay) the target tissue to assess the stability and maintenance of expression of the exogenous genetic material in the target cell population. A diminution of reporter gene #1 expression compared to reporter gene #2 expression can indicate either immune attack against transduced cells or transduction of short-lived, differentiated cells. An exemplary procedure for processing a target tissue to which a β -galactosidase reporter gene expression has been delivered to determine whether the reported gene is expressed therein includes: a) snap freezing the tissue in isopentane chilled with liquid nitrogen, b) mounting the tissue on cryomold (Tissue -Teck, Miles, Elkhart, IN) using OCT and freezing, c) cutting the frozen tissue with a microtome at -20°C into 10 μm sections, d) staining the tissue for β -galactosidase reporter gene expression with X-gal (1mg/ml) (a reporter gene substrate, Boehringer Mannheim, Indianapolis, IN), potassium ferro- and ferricyanide (35mM each) in phosphate buffered saline solution. To detect expression of a therapeutic polynucleotide, the above-described procedure is used with the exception that immunostaining using an antibody to detect the therapeutic polynucleotide expression product is used in place of the reporter gene substrate assay.

In the following example, delivery of the CMV β -gal gene-containing galactosylated and ungalactosylated phage particles *in vitro* to HepG2 cells and *in vivo* (Example IV) to liver cells of mice was measured. Histochemical staining of HepG2 cells using X-gal was used to measure expression of the β -gal reporter gene. The results showed a field of Hep G2 cells expressing the β -gal gene, as indicated by the appearance of positive cells that stained darkly following incubation with the substrate X-gal. The control experiment (ungalactosylated phage particles) did not show positive cells that stained darkly following incubation with the substrate X-gal. These results demonstrate that the galactose-labeled phage particles were selectively internalized by the murine liver cells, presumably via receptor-mediated endocytosis.

Example IV**The targeted delivery of a modified bacteriophage of the invention to hepatocytes in the liver of live mice *in vivo*.**

For the *in vivo* experiment, 10^{10} phage particles, either modified with an average of 50 galactose residues/phage particle or unmodified (control), were injected into mice intraperitoneally. Three days after injection, mice were sacrificed and liver and kidney tissue biopsies were prepared. Frozen sections were cut and fixed with 1% glutaraldehyde and subsequently stained with X-gal. After staining, tissue sections were analyzed by light microscopy. Unmodified phage (control) injection did not result in β -gal expression in either liver or kidney tissue sections that were stained with X-gal as detailed above. In contrast, injection of galactosylated phage resulted in detectable β -gal staining in hepatocytes but not in any cells on kidney tissue sections from the same mouse. Careful analysis of 40 serial sections of liver removed from the mouse exposed to the galactosylated phage demonstrated that approximately 0.5 to 1% of hepatocytes stained with X-gal and thus, effectively were transduced by the recombinant bacteriophage vector. While this example shows that intraperitoneal administration could be used successfully to transduce hepatocytes *in vivo*, one skilled in the art reasonably would believe that alternative routes of administration (such as those described in the detailed description), as well as the further inclusion of endosomal lysis signals and/or nuclear localization signals also can be used to successfully transduce mammalian target cells *in vivo* and *in vitro*.

Example V**Construction of Bacteriophage Lambda vectors with Modified Tail Proteins.**

As described above, bacteriophage with wild type packaging proteins and the beta-galactosidase gene are not effective genetic vectors for transducing Hep G2 cells. However, chemical modification of the surface proteins *in vitro* with galactose such that it will bind to and be internalized by the galactose-receptor on the Hep G2 cell surface does result in an effective vector. The galactose-asialoglycoprotein ligand-receptor interaction and subsequent internalization via receptor mediated endocytosis have been described in detail in the literature. Accordingly, the above-noted results, together with the literature reports documenting the mechanism of asialoglycoprotein-mediated transport, supports our hypothesis that the transfer of genetic material from the above-described modified bacteriophages to a mammalian cell target occurs via receptor-mediated endocytosis.

Thus, we believe that the mechanism by which modified bacteriophage vectors transfer genetic material to mammalian target cells is fundamentally different from the mechanism by which wild type bacteriophages transduce their natural prokaryotic hosts and that functional bacteriophage tail proteins are not essential for mammalian target cell transduction using the bacteriophages of the invention. This hypothesis is confirmed using the screening methods described below.

Using standard methods, endocytosis inhibition agents (e.g., colchicine, cytochalasin B and D, and monodansylcadaverine) are used to arrest cell-mediated endocytosis in a mammalian cell and, thereby, select for bacteriophages whose mechanism of transfer does not depend on injection of genetic material into the mammalian cell as is observed in the naturally occurring bacteriophage transduction of prokaryotic hosts. Alternatively, antibodies that bind to bacteriophage tail proteins and block their function can be used to demonstrate that wild type tail function is not required for the transfer of the bacteriophage genome to the host cell nucleus. The methods also are useful for identifying target cells that contain an appropriate receptor in sufficient quantity to internalize the preferred bacteriophage vectors via receptor-mediated endocytosis. The screening assays disclosed herein demonstrate that bacteriophage vectors which include one or more non-functional tail protein(s) and a standard genomic expression marker, e.g. beta-galactosidase or other detectable protein, are incapable of transferring genetic material to the target cells in the presence of the endocytosis inhibition agents but are capable of transduction when contacted with the target cells in the absence of such inhibition agents. Thus, the assay is useful for identifying novel bacteriophages which are structurally and functionally distinct from naturally-occurring bacteriophages and modified bacteriophage such as those described in PCT publication no. WO 96/21007, entitled, "Bacteriophage-mediated Gene Transfer Systems Capable of Transfecting Eukaryotic Cells", applicant Chiron Viagene, Inc. which report modified bacteriophages that inject their genetic contents into mammalian target cells.

Standard recombinant methods and screening technology are used to prepare a preferred bacteriophage vector with genetically modified tail protein genes that either contain amber mutations or sequences that enhance binding of the bacteriophage vector to the mammalian cell surface and subsequent internalization via receptor-mediated

endocytosis. Exemplary essential tail proteins in the lambda phage that can be modified to prepare preferred embodiments of the invention include: H, J, M proteins. As used herein, "essential tail proteins" refers to those proteins that are essential for facilitating the injection of the bacteriophage genetic material into its natural prokaryotic host. Although Applicants doubt the likelihood that bacteriophage can be selected or designed to have tail proteins that are capable of facilitating the injection of the bacteriophage genetic material into a eukaryotic host, the phrase "essential tail proteins" is also meant to embrace the tail proteins of such hypothetical eukaryotic cell-injecting bacteriophage. One or more of these or other tail proteins can be rendered non-functional (i.e., incapable of facilitating the injection of the genetic material into the host cell) using recombinant, mutagenesis, and/or chemical methods in accordance with procedures known to one of ordinary skill in the art. Such procedures can, of course, be applied to the preparation of other types of modified bacteriophages by, e.g., identifying the essential tail proteins of one or more bacteriophages in Table 1 and modifying the essential tail proteins as described herein.

The modifications to the essential tail protein(s) renders the bacteriophage incapable of mediating the transfer of genetic material into a mammalian host via an injection mechanism analogous to that responsible for the naturally occurring bacteriophage transduction of prokaryotic hosts. Preferably, such modification(s) of the tail proteins further render the bacteriophage incapable of infecting and/or propagating within its natural prokaryotic host (Su^0 E.Coli bacteria). Accordingly, such tail mutant bacteriophage vectors are packaged in vitro using specific purified protein packaging extracts and recombinant bacteriophage genomes or in Su^+ E. Coli bacteria (for amber mutant containing bacteriophage vectors). The benefit of such preferred bacteriophage vectors is that these vectors are incapable of propagating in the host organism's natural flora.

In certain embodiments, the tail proteins are modified so that they function to properly package the virus' genome but have lost their ability to mediate injection across bacterial cell membranes. Phages containing these modified tail-proteins require packaging in a cell-free system as described above.

In yet another embodiment, the mutations in the tail proteins are temperature sensitive such that at temperatures other than mammalian physiologic temperature, the tail

Example VI

BNSDOCID: <WO 9805344A1 | >

The following assay is useful for selecting target cells and bacteriophage vectors that depend on receptor mediated endocytosis for transfer of the bacteriophage genome to the target cell nucleus. The modified bacteriophages are grown to high titer 10^{12} to 10^{13} by standard methods employing either endogenous packaging in a prokaryotic host or in vitro packaging with proteins extracted from appropriately engineered lysogen strains. Additionally or alternatively, these modified bacteriophages may be modified chemically after packaging using standard methods. The coding sequence for beta-galactosidase or other easily detectable gene expression indicator(s) is incorporated into the genome of the test bacteriophages using standard procedures. At preferable multiplicities of infection of 1 to 100, purified bacteriophage is then added to sub-confluent cultures on cover slips of mammalian test cells that bear the receptor to which a modified bacteriophage has been targeted. One example of such a test cell line is HepG2 cells in which the asialoglycoprotein mediated endocytosis of galactose bearing oligosaccharides and macromolecules is well studied (described above).

The screening assay is performed in the presence and absence of endocytosis inhibitors. Preferably, prior to addition of the modified bacteriophage, inhibitors of endocytosis are added to the cell medium containing the target cells either singly or in various combinations at concentrations such that receptor mediated endocytosis is optimally inhibited without irreversibly injuring the test target cells. Exemplary endocytosis inhibitors include colchicine, taxol, monodansylcadaverine, cytochalasin B, or cytochalasin D. As a control, cells not treated with endocytosis inhibitors are infected with test bacteriophages in parallel.

The test cells are incubated with bacteriophage for about 48 hours or other suitable time to allow transduction and expression. At various time points during this period, the plated cells are fixed for x-gal staining, immunostaining, in situ hybridization, electron microscopy or other standard methods that are known to one skilled in the art for detecting the inserted exogenous polynucleotide or its expression product. X-gal staining is used to indicate whether or not the transduced genetic material (including a beta-galactosidase marker) is expressed in the test cells. Alternatively, immunostaining with specific antibodies or anti-sense in situ hybridization probes employed to detect either the translation or transcription of a gene inserted into the vector's genome. Additionally or

alternatively, antibodies with specific affinity for the modified bacteriophage surface protein(s) are used to detect the localization of viral coat proteins within test cells, including organelles within the test cells, using confocal immunofluorescence microscopy. Optionally, transmission electron microscopy is employed to determine the sub cellular localization of modified bacteriophages at various time points after introduction of the modified bacteriophage into the test cell media.

It is anticipated that less than about 5% of target cells that are contacted with the endocytosis inhibitor(s) demonstrate internalization and/or expression of the indicator gene. In addition, immunostaining with anti-coat antibodies of sections of test cells that have been exposed to endocytosis inhibitors and fixed directly following incubation with bacteriophage detect the presence of viral coat proteins in a sub-plasma membrane location, thus, indicating entrapment of these bacteriophages within endocytic vesicles prevented from fusion with lysosomes and normal trafficking. In contrast, the bacteriophage-infected test cells in which receptor-mediated endocytosis is not inhibited exhibit expression of the indicator gene in greater than about 15% of the test cells. Furthermore, immunostaining uninhibited test cell sections for coat proteins demonstrate a predominance of sub plasma membrane localization. These results are surprising and unexpected in view of the internalization mechanism proposed in PCT publication no. WO 96/21007 for bacteriophage transfer of genetic material to a mammalian cell.

Example VII:

Construction of a modified bacteriophage vector which displays cyclic RGD ligand on its surface

We have designed a bacteriophage genome which appends, in frame, the coding sequence cyclic RGD ligand 3' to the coding sequence of the native D-gene. This bacteriophage genome was packaged *in vitro* and propagated in *E. Coli* so as to produce productive phage which contains the D-gene-RGD fusion protein product on its surface. The effect of displaying this cyclic RGD ligand on the surface is to promote uptake of this bacteriophage by mammalian cells bearing the alpha-5/beta-1 integrin receptor via cell-mediated endocytosis. This modified bacteriophage is referred to as lambda DASH II-RGD. Using a beta-galactosidase reporting construct, we have found the lambda DASH II-RGD to infect 30-40% of cultured monolayer bovine endothelial cells at a multiplicity

of infection of 1000 phage particles per cell. The sequences of the RGD primers are shown in SEQ ID NOS. 18, 19, 20, and 21.

Example VIII:

Delivery and expression of human factor VIII/von Willebrand factor containing vector

As further enabling proof of our technology, we have constructed a bacteriophage genome which contains the following functional elements in 5' to 3' tandem array: CMV promoter-human factor VIII cDNA (Seq ID No. 15) - internal ribosomal entry site (IRES)-human von Willebrand factor cDNA (Seq. ID No. 17). The IRES cassette was obtained from Clontech (Palo Alto, CA). The total size of this linear DNA construct is 20.6 kilobases. As described in the detailed description of the invention, our FVIII/vWF construct has been spliced into the multiple cloning site of the bacteriophage lambda DASH II-RGD genome via blunt ended ligation (see included sequences and construct diagram).

This resulting genome has been efficiently packaged and propagated to titers of 10^{11} to 10^{12} phage particles per 2 liter broth culture. We have infected subconfluent monolayer cultures of bovine endothelial cells with this FVIII-vWF containing vector. Using a standard assay for factor VIII activity (COATEST VIII: C4-Chromogenenix Mölndal, Sweden), we have measured expression of factor VIII at 48 hours after infection. A 24 hour secretion study revealed that these transduced endothelial cells produce 56 mU/24 hours/10 cm culture plate (approximately 5×10^5 cells). This secretion rate is 8 fold greater than that reported for a transfected COS cell line (Toole et al., Nature, 312, 1984, p. 342-7).

Example IX: Delivery and expression of a murine dystrophin containing vector

As described in the examples of the patent application, we have obtained a 20.5 kilobase insert from Dr. Jeffrey Chamberlain (Michigan University) containing the following functional elements in tandem oriented 5' to 3': muscle creatine kinase promoter - murine full length dystrophin cDNA (Seq ID No. 17). We, in turn, have spliced this dystrophin insert into lambda DASH II-RGD via blunt end ligation into the multiple cloning site. The resulting recombinant bacteriophage genome has been packaged and

propagated efficiently to titers of 10^{11} to 10^{12} phage particles per 2 liter broth culture.

To test the expression capacity of this dystrophin bacteriophage vector, we injected 50 microliters of 10^{10} phage particles/mL into the gastrocnemius muscle of a dystrophin-deficient mouse (*mdx*). The phage vector was co-injected with a sublethal dose of India ink. This permitted accurate localization of the myocytes in proximity to the needle tract. Employing a polyclonal antibody against dystrophin (Cox et al. Nature, 264, 1993, 725-9), we have been able to demonstrate by immunofluorescence restoration of perimyocyte dystrophin expression. As previously reported (*Ibid.*), immunofluorescence study of the control uninjected *mdx* mouse muscle with the same anti-dystrophin antibody did not demonstrate specific perimyocyte immunoreactivity.

Each of the references, patents and patent publications disclosed in this document is incorporated in its entirety herein by reference.

While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is intended that such modification, changes and equivalents fall within the scope of the following claims.

The Tables are presented below and are followed by the Sequence Listing and what is claimed:

Table 1 Exemplary Bacteriophages

Lambda phage, p1 phage, T even and T odd phages (e.g., T1, T2, T3, T4, T5, T6 and T7); P2; P4; Mu; PM2; N4; SPO1; PBS1; PBS2; 29; SPP1; 6; PR4; PRD1; AP50; DS6A; I3; NS11; Dp-1; MVL2; CP-1; 434; cbk; G; D108; and P7.

Table 2

Exemplary Human Gene Therapy Protocols Approved by RAC

Disease	Gene Therapy Treatment	RAC OK
Severe combined immune deficiency (SCID) due to adenosine deaminase (ADA) deficiency	Autologous lymphocytes transduced with human ADA gene	7/31/90

Advanced cancer	Tumor-infiltrating lymphocytes transduced with tumor necrosis factor gene	7/31/90
Advanced cancer	Immunization with autologous cancer cells transduced with tumor necrosis factor gene	10/07/91
Advanced cancer	immunization with autologous cancer cells transduced with interleukin-2 gene	10/07/91
Familial hypercholesterolemia	<i>Ex vivo</i> gene therapy	10/08/91
Malignancy	<i>In vivo</i> gene transfer into tumors	2/10/92
Cancer	Gene transfer	2/10/92
Relapsed/refractory neuroblastoma	Cytokine-gene modified autologous neuroblastoma cells (Phase I study)	6/01/92
Brain tumors	Intratumoral transduction with thymidine kinase gene and intravenous ganciclovir	6/01/92
Metastatic melanoma	Immunization with HLA-A2 matched allogeneic melanoma cells that secrete interleukin-2	6/02/92
Advanced renal cell carcinoma	Immunization with interleukin-2 secreting allogeneic HLA-A2 matched renal-cell carcinoma cells	6/02/92
Cancer	Interleukin-4-gene modified antitumor vaccine (pilot study)	9/15/92
Cystic fibrosis	Replication deficient recombinant adenovirus carrying cDNA of normal human cystic fibrosis transmembrane conductance regulator (CFRT) gene; single administration to the lung (Phase I study)	12/03/92
Cystic fibrosis	El-deleted adenovirus vector for delivering CFTR gene (Phase I study)	12/03/92

Disease	Gene Therapy Treatment	RAC OK
Cystic fibrosis	Adenovirus vector used for delivering CFTR gene to nasal epithelium	12/04/92
Recurrent glioblastoma (brain tumor)	<i>In vivo</i> tumor transduction using herpes simplex thymidine kinase gene/ganciclovir system	3/01/93
Metastatic renal cell carcinoma	Injection of non-replicating autologous tumor cells prepared +/- granulocyte-macrophage colony stimulating factor transduction (Phase I study)	3/01/93
Cystic fibrosis	Use of replication deficient recombinant adenovirus vector to deliver human CFTR cDNA to the lungs (Phase I study)	3/02/93
Cystic fibrosis	Use of EI-deleted adenovirus for delivery of CFTR gene to nasal cavity (Phase I study)	3/02/93
Disseminated malignant melanoma	Human gamma-interferon transduced autologous tumor cells (Phase I study)	6/07/93
Ovarian cancer	Use of modified retro viruses to introduce chemotherapy resistance sequences into normal hematopoietic cells for chemoprotection (pilot study)	6/07/93
Cancer	Immunotherapy by direct gene transfer into tumors	6/07/93
Gaucher's disease	<i>Ex vivo</i> gene transfer and autologous transplantation of CD34 + cells	6/07/93
Gaucher's disease	Retro viral-mediated transfer of cDNA for human glucocerebrosidase into hematopoietic stem cells	6/07/93
Asymptomatic patients infected with HIV-1	Murine Retro viral vector encoding HIV-1 genes [HIV-IT(V)]	6/07/93

SUBSTITUTE SHEET (RULE 26)

AIDS	Effects of a transdominant form of <i>rev</i> gene on AIDS intervention	6/07/93
Recurrent pediatric malignant astrocytomas	<i>In vivo</i> tumor transduction with herpes simplex thymidine kinase gene	6/08/93
Advanced cancer	Human multiple-drug resistance (MDR) gene transfer	6/08/93
Brain tumors	Episome-based antisense cDNA transcription of insulin-like growth factor I	6/08/93
Small-cell lung cancer	Cancer cells transfected with and expressing interleukin-2 gene (Phase I study)	9/09/93

Disease	Gene Therapy Treatment	RAC OK
Breast cancer (post-chemotherapy)	Retro viral mediated transfer of the human MDR gene into hematopoietic stem cells (autologous transplantation)	9/09/93
Recurrent pediatric brain tumors	Intra-tumoral transduction with thymidine kinase gene and intravenous administration of ganciclovir	9/09/93
Malignant melanoma	Immunization with interleukin-2 secreting allogeneic human melanoma cells	9/10/93
HIV infection	Autologous lymphocytes transduced with catalytic ribozyme that cleaves HIV-1 RNA (Phase I study)	9/10/93
Metastatic melanoma	Genetically engineered autologous tumor vaccines producing interleukin-2	9/10/93
Leptomeningeal carcinomatosis	Intrathecal gene therapy	12/02/93
Colon carcinoma	Injection with autologous irradiated tumor cells and fibroblasts genetically modified to secrete interleukin-2	12/2/93
Gaucher's disease	Retro virus-mediated transfer of cDNA for human glucocerebrosidase into peripheral blood repopulating patients' cells	12/3/93
HIV infection	Murine Retro viral vector encoding HIV-IT(V) genes (open label Phase I/II trial)	12/03/93
Advanced (stage IV) melanoma	Induction of cell-mediated immunity against tumor-associated antigens by B7-transfected lethally irradiated allogeneic melanoma cell lines (Phase I study)	12/03/93

SUBSTITUTE SHEET (RULE 26)

-55/2-

Advanced colorectal carcinoma	Immunotherapy by direct gene transfer into hepatic metastases (Phase I study)	12/03/93
Melanoma	Adoptive immunotherapy with activated lymph node cells primed <i>in vivo</i> with autologous tumor cells transduced with interleukin-4 gene	12/03/93
Cystic fibrosis	Cationic liposome-mediated transfer of CFTR gene into nasal airway (Phase I study)	12/03/93
Cystic fibrosis	Adenovirus-mediated transfer of CFTR gene to the nasal epithelium and maxillary sinus	12/03/93
Pediatric neuroblastoma	Immunization with gamma-interferon transduced neuroblastoma cells (<i>ex vivo</i>) (Phase I)	3/03/94

Table 3

Preferred Therapeutic Polynucleotides and Corresponding Medical Conditions

Dystrophin-Duchenne muscular dystrophy
 5 Globin gene complex-Hemoglobinopathies, e.g. sickle cell anemia, thalassemias
 Clotting factor VIII-Hemophilia A
 von Willebrand's factor-von Willebrand's disease
 Collagen type VII-Epidermolysis bullosa dystrophica
 Combinations of cyclin dependent kinase inhibitors, and other cell cycle inhibitors,
 10 e.g. p15, p16, p18-Neoplastic processes, e.g. melanoma
 Fibrillin-Marfan's syndrome
 Polypeptide antigens-Vaccines for tumors, infectious agents
 Combinations of cytokines or co-stimulatory immune modulators, e.g., IL-1, IL-2, IL12,
 GM-CSF, TNF α , IL4, B7-Neoplastic processes
 15 Thymidine kinase-Suicide gene for neoplastic, hyperplastic or hypertrophic processes
 Combinations of ribozymes-Targeted against disease predisposing MHC genes or against
 disease associated messenger RNAs of viral origin, e.g. E6, E7 oncoproteins
 in HPV, reverse transcriptase in HIV
 Individual or combinations of chemotherapy resistance genes to protect bone marrow
 20 stem cells from chemotherapy regimens
 Polypeptide antigens in conjunction with tolerance inducing sequences, e.g. ribozyme
 against B7-1-Treatment for autoimmune disease, e.g. rheumatoid arthritis,
 psoriasis, multiple sclerosis, alopecia areata
 Combinations of ribozyme(s), antisense RNA(s), or polypeptide coding sequences(s) for
 25 biopolymers that interfere with human viral infections, e.g. HIV, CMV, Hepatitis
 B and C, Herpes Simplex I, II, HHV-8, EBV, HTLV-I
 Combinations of ribozyme(s), antisense RNA(s), or polypeptide coding sequences(s) for
 biopolymers that interfere with chronic bacterial or parasitic infections, e.g.
 leprosy, tuberculosis, antibiotic resistant bacteria (e.g., MRSA, VRE),
 30 trypanosomiasis, filaraisis, and the like

Table 4

Exemplary Target Cells

Hepatocytes
5 Melanocytes
Keratinocytes
Myocytes
Adipocytes
Hematopoietic cells, e.g. lymphocytes, erythrocytes, leukocytes, monocytes, progenitor
10 and stem cells
Neurons
Glial cells
Antigen presenting cells, e.g. macrophages, B-cells, Langerhan's cells
Chondrocytes
15 Osteocytes
Osteoclasts
Endothelial cells
Phagocytes
Fibroblasts
20 Smooth muscle cells
Renal tubule cells
Mesangial cells
Thymocytes
Bronchopulmonary, gastrointestinal, breast, genitourinary, corneal, renal ductular
25 epithelial cells
Endocrine, exocrine gland cells
Plasma cells
Mast cells
Lens epithelial cells
30 Retinal epithelial cells
Malignant cells of any derivation
Placental cells
Gonadal cells
Embryonic cells
35 Zygotes

Table 5

Exemplary Promoters and EnhancersConstitutive

Phosphoglycerokinase

Long terminal repeat (LTR) of retroviruses, e.g. Moloney murine leukemia virus,
Rous sarcoma virus

Cytomegalovirus promoter

Hematopoietic cells

Promoters

c fms (monocytes, trophoblasts)

T-cell receptor

Enhancers

Immunoglobulin heavy chain

Locus control region of the globin gene complex CD2

Hepatocytes

Promoters

Albumin

 α -1-antitrypsin

Pyruvate kinase

Phosphoenol pyruvate carboxykinase

Transferrin

Transthyretin

 α -fetoprotein α -fibrinogen β -fibrinogen

Hepatitis B

Enhancers

Hepatitis B

Tyrosine aminotransferase

Cardiac myocytes

Promoter

Myosin light chain-2

 β -myosin heavy chain (cardiac and slow twitch skeletal) α -cardiac myosin heavy chain

Cardiac alpha actin

Enhancer

 β -myosin heavy chainFibroblasts

Promoter

Collagen alpha-2 (I)

Elastin (fibroblasts and smooth muscle cells)

Neurons

Promoter

Peripheral myelin protein-22

Adipocytes

Promoter

Lipoprotein lipase

Aromatase cytochrome P450 (adipocytes, brain, ovary)

Thyroid

Promoter

Thyroglobulin

Lens epithelium

Promoter

Crystallin

Breast epithelium

Promoter

Milk protein gene

Skeletal muscle

Promoter

Glut-4

Muscle creatine kinase (skeletal and cardiac muscle)

Enhancer

Muscle creatine kinase (skeletal and cardiac muscle)

Urinary bladder

Promoter

UroplakinII

Keratinocyte

Promoter

Keratin 14

Keratin 10

Involucrin

Melanocyte

Promoter

Tyrosinase

Non specific enhancer elements

SV40

CMV

LTR

Inducible or repressible promoter systems

Estrogen-Gal4 inducible system

RU486-Gal4 inducible system

Tetracycline inducible system

IPTG system

Metallothionein

Tetracycline repressible system

Table 6
Exemplary Receptors and Preferred Ligands

Hepatic receptors

hyaluronic acid
collagen
N-terminal propeptide of collagen type III
mannose/N-acetylglucosamine
complement
asialoglycoprotein
tissue plasminogen activator
low density lipoprotein
insulin
ceruloplasmin
enterokinase
carcinoembryonic antigen
apamin
galactose/lactose

Growth Factor/Cytokine receptors

hepatocyte growth factor
epidermal growth factor
insulin-like growth factor I, II
interleukin-1 a/b
interleukin-2, IL-7, IL-4
 γ -interferon
 β -interferon
keratinocyte growth factor
TNF-R p55

Hormone receptors

prolactin
thyroglobulin
growth hormone
insulin
glucagon
leutinizing hormone
human choriogonadotrophic hormone

Nerve cell receptors

neurotensin

Antigen presenting cell receptors

immunoglobulin G-Fc receptor

Kidney cells
angiotensin II
vasopressin

5 Bone marrow receptors
c kit
CD-34

10 Keratinocyte and skin fibroblast receptors
very low density lipoprotein
low density lipoprotein
integrins that bind to RGD bearing polypeptides
collagen
laminin

15 Placental receptors
hemopexin
immunoglobulin G-Fc
low density lipoprotein
20 transferrin
alpha2-macroglobulin
ferritin
insulin
gamma-interferon
25 epidermal growth factor
insulin-like growth factor

Muscle cell receptors
insulin
30 very low density lipoprotein

Gut epithelium
cobalamin-intrinsic factor
heat stable enterotoxin of E. Coli

35 Breast epithelium
heregulin
prolactin

40 Melanocytes
c kit

Miscellaneous
folate
45 cobalamin (B12)

Preferred ligands

low density lipoprotein (apoprotein B100)
very low density lipoprotein (apoprotein E)
galactose
c kit ligand
transferrin
insulin
heregulin
RGD or RGD-containing polypeptides

Table 7Exemplary Antigens to Induce or Enhance an Immune Response

Melanoma or other tumor specific antigens;
leishmaniasis antigens;
helicobacter pylori specific antigens (e.g., urease B);
hepatitis B antigens;
hepatitis C antigens;
Herpes simplex antigens;
HIV antigens;
Tuberculosis antigens;
cytomegalovirus antigens;
lyme disease antigens;
malaria antigens;
respiratory syncytial virus antigens;
leprosy antigens;
toxoplasmosis antigens;
pneumocytis carinii antigens;
schistosomiasis antigens;
chlamydial antigens;
HTLV-1 antigens;
enterococcal antigens (e.g., VRE);
gonococcal antigens;
treponemal antigens;
clostridium difficile antigens;
Staphylococcus aureus antigens (e.g., MRSA);
trypanosomal antigens;
filarial antigens;
salmonella antigens;
shigella antigens;
pneumococcal antigens (e.g., penicillin resistant strains);
pseudomonal antigens

Table 8

Advantages of the Targeted Phage Vectors Over
the Vectors Currently Used in Gene Therapy

	Size Constraints	Specificity of Targeting	Immunogenicity/ Toxicity	Sustained/High/Low/ Controlled Expression
Retrovirus Vector	7Kb	none	none	low, uncontrolled transient transfection
Adenovirus Vector	7Kb	none	high immunogenicity	low, uncontrolled transient transfection
Liposome	none	none	toxic at high doses	low, uncontrolled transient transfection
Phage Vectors	bacteriophage lambda DASH II (up to 23Kb); Minimal lambda cassette (up to 50 kb); P1 (up to 95 Kb)	yes	none	controlled high persistent expression

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: BRIGHAM AND WOMEN'S HOSPITAL, INC.
- (B) STREET: 75 FRANCIS STREET
- (C) CITY: BOSTON
- (D) STATE: MASSACHUSETTS
- (E) COUNTRY: UNITED STATES OF AMERICA
- (F) POSTAL CODE: 02115

(i) APPLICANT/INVENTOR:

- (A) NAME: SARKAR, SAUMYENDRA N.
- (B) STREET: 102 QUEENSBURY STREET
- (C) CITY: BOSTON
- (D) STATE: MASSACHUSETTS
- (E) COUNTRY: UNITED STATES OF AMERICA
- (F) POSTAL CODE: 02115

(i) APPLICANT/INVENTOR:

- (A) NAME: KUPPER, THOMAS S.
- (B) STREET: 8 SURREY LANE
- (C) CITY: WESTON
- (D) STATE: MASSACHUSETTS
- (E) COUNTRY: UNITED STATES OF AMERICA
- (F) POSTAL CODE: 02193

(i) APPLICANT/INVENTOR:

- (A) NAME: DUBIN, DANIEL B.
- (B) STREET: 99 POND AVE
- (C) CITY: BROOKLINE
- (D) STATE: MASSACHUSETTS
- (E) COUNTRY: UNITED STATES OF AMERICA
- (F) POSTAL CODE: 02146

(ii) TITLE OF THE INVENTION: BACTERIOPHAGE-MEDIATED GENE
THERAPY

(iii) NUMBER OF SEQUENCES: 21

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: WOLF, GREENFIELD & SACKS, P.C.
- (B) STREET: 600 ATLANTIC AVENUE
- (C) CITY: BOSTON
- (D) STATE: MASSACHUSETTS

-66-

(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE: 02210

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/693,865
(B) FILING DATE: 08-MAY-1996

(A) APPLICATION NUMBER: US 08/814,859
(B) FILING DATE: 11-MAR-1997

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Plumer, Elizabeth R.
(B) REGISTRATION NUMBER: 36,637
(C) REFERENCE/DOCKET NUMBER: B0801/7059WO

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-720-3500
(B) TELEFAX: 617-720-2441

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacteriophage lambda

SUBSTITUTE SHEET (RULE 26)

-67-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATACCGAGGG CTGCAGTGTA CA

22

5 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

20 (A) ORGANISM: Bacteriophage lambda

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTCTTTCAAT TGGGGAGGCA AAACGATGCT GATTGCCGTT C

41

25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

30 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

40

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

45 TTGCCTCCCC AATTGAAAGA G

21

(2) INFORMATION FOR SEQ ID NO:4:

SUBSTITUTE SHEET (RULE 26)

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGATGAAGG GTAAAGTTAT TTGCGTTTTT TTTTCGGCGG GGTCTCCAT AAATTCAATC

60

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacteriophage lambda

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAACTTTACC CTTCACTACT AAAGGCC

27

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

SUBSTITUTE SHEET (RULE 26)

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacteriophage lambda

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAACGTACAG CGCCATGTTT ACCAG

25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Pro Lys Lys Lys Arg Lys Val

1

5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Ser Arg Lys Arg Pro Arg Pro

1

5

(2) INFORMATION FOR SEQ ID NO:9:

SUBSTITUTE SHEET (RULE 26)

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys Lys Lys Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Arg Thr Thr Lys Gly Lys Arg Lys Arg Ile Asp Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

SUBSTITUTE SHEET (RULE 26)

Ala Ala Lys Arg Val Lys Leu Asp
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Ser Ser Lys Arg Pro Arg Pro
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Lys Ile Arg Leu Arg Pro Gly Gly Lys Lys Lys Tyr Lys Leu Lys His
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 300 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

SUBSTITUTE SHEET (RULE 26)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

5	Met	Thr	Ser	Arg	Arg	Ser	Val	Lys	Ser	Gly	Pro	His	Glu	Val	Pro	Arg	1	5	10	15
	Gln	Glu	Tyr	Glu	Asp	Leu	Tyr	Tyr	Thr	Pro	Ser	Ser	Gly	Met	Ala	Ser	20	25	30	
10	Pro	Gln	Ser	Pro	Pro	Gln	Thr	Ser	Arg	Arg	Gly	Ala	Leu	Gln	Thr	Arg	35	40	45	
	Ser	Arg	Gln	Arg	Gly	Glu	Val	Arg	Phe	Val	Gly	Tyr	Asp	Glu	Ser	Asp	50	55	60	
15	Tyr	Ala	Leu	Tyr	Gly	Gly	Ser	Ser	Ser	Glu	Asp	Asp	Glu	His	Pro	Glu	65	70	75	80
	Pro	Pro	Thr	Arg	Arg	Pro	Val	Ser	Gly	Ala	Val	Ala	Ser	Gly	Pro	Gly	85	90	95	
20	Pro	Ala	His	Ala	Pro	Pro	Pro	Pro	Ala	Gly	Ser	Gly	Gly	Ala	Gly	Arg	100	105	110	
	Thr	Pro	Thr	Thr	Ala	Pro	Arg	Ala	Pro	Arg	Thr	Gln	Arg	Val	Ala	Thr	115	120	125	
25	Lys	Ala	Pro	Ala	Ala	Pro	Ala	Ala	Glu	Thr	Thr	Arg	Gly	Arg	Lys	Ser	130	135	140	
30	Ala	Gln	Pro	Glu	Ser	Ala	Ala	Leu	Pro	Gln	Ala	Pro	Ala	Ser	Thr	Ala	145	150	155	160
	Arg	Thr	Arg	Ser	Lys	Thr	Pro	Ala	Gly	Gly	Leu	Ala	Arg	Lys	Leu	His	165	170	175	
35	Glu	Ser	Thr	Ala	Pro	Pro	Asn	Pro	Asp	Ala	Pro	Val	Val	Thr	Pro	Arg	180	185	190	
40	Val	Ala	Gly	Phe	Asn	Lys	Arg	Val	Cys	Ala	Ala	Val	Gly	Arg	Leu	Ala	195	200	205	
	Ala	Met	His	Ala	Arg	Met	Ala	Ala	Val	Gln	Leu	Val	Val	Asp	Met	Ser	210	215	220	
45	Arg	Pro	Arg	Ile	Asp	Glu	Asp	Ile	Asn	Glu	Leu	Leu	Gly	Ile	Thr	Thr	225	230	235	240

SUBSTITUTE SHEET (RULE 26)

Ile Arg Val Thr Val Cys Glu Gly Lys Asn Leu Leu Gln Arg Ala Asn
245 250 255

Glu Val Asn Pro Asp Val Val Gln Asp Val Asp Ala Ala Thr Ala Thr
260 265 270

Arg Gly Arg Ser Ala Ala Ser Arg Pro Thr Glu Arg Pro Arg Ala Pro
275 280 285

Ala Pro Ser Ala Ser Arg Pro Arg Arg Pro Val Glu
290 295 300

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9009 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

	CAGTGGGTAA GTTCCTTAAA TGCTCTGCAA AGAAATTGGG ACTTTTCATT AAATCAGAAA	60
	TTTTACTTTT TTCCCCTCCT GGGAGCTAAA GATATTTTAG AGAAGAATTA ACCTTTTGCT	120
	TCTCCAGTTG AACATTTGTA GCAATAAGTC ATGCAAATAG AGCTCTCCAC CTGCTTCTTT	180
30	CTGTGCCTTT TGCGATTCTG CTTTAGTGCC ACCAGAAGAT ACTACCTGGG TGCAGTGGAA	240
	CTGTCAATGG ACTATATGCA AAGTGATCTC GGTGAGCTGC CTGTGGACGC AAGATTTTCT	300
	CCTAGAGTGC CAAAATCTTT TCCATTCAAC ACCTCAGTCG TGTACAAAAA GACTCTGTTT	360
	GTAGAATTCA CGGTTTACCT TTTCAACATC GCTAAGCCAA GGCCACCCTG GATGGGTCTG	420
	CTAGGTCCTA CCATCCAGGC TGAGGTTTAT GATACAGTGG TCATTACACT TAAGAACATG	480
35	GCTTCCCATC CTGTCAGTCT TCATGCTGTT GGTGTATCCT ACTGGAAAGC TTCTGAGGGA	540
	GCTGAATATG ATGATCAGAC CAGTCAAAGG GAGAAAGAAG ATGATAAAGT CTTCCTGGT	600
	GGAAGCCATA CATATGTCTG GCAGGTCCTG AAAGAGAATG GTCCAATGGC CTCTGACCCA	660
	CTGTGCCTTA CCTACTCATA TCTTTCTCAT GTGGACCTGG TAAAAGACTT GAATTCAGGC	720
	CTCATTTGGAG CCCTACTAGT ATGTAGAGAA GGGAGTCTGG CCAAGGAAA GACACAGACC	780
40	TTGCACAAAT TTATACTACT TTTTGCTGTA TTGTATGAAG GGAAAAGTTG GCACTCAGAA	840
	ACAAAGAAGT CCTTGATGCA GGATAGGGAT GCTGCATCTG CTCGGGCCTG GCCTAAATG	900
	CACACAGTCA ATGGTTATGT AAACAGGTCT CTGCCAGGTC TGATTGGATG CCACAGGAAA	960
	TCAGTCTATT GGCATGTGAT TGGAAATGGGC ACCACTCCTG AAGTGCACTC AATATTCCTC	1020
	GAAGGTCACA CATTTCTTGT GAGGAACCAT CGCCAGGCGT CCTTGGAAAT CTCGCCAATA	1080
45	ACTTTCCTTA CTGCTCAAAC ACTCTTGATG GACCTTGGAC AGTTTCTACT GTTTTGTCTAT	1140
	ATCTCTTCCC ACCAACATGA TGGCATGGAA GCTTATGTCA AAGTAGACAG CTGTCCAGAG	1200
	GAACCCCAAC TACGAATGAA AAATAATGAA GAAGCGGAAG ACTATGATGA TGATCTTACT	1260

	GATTCTGAAA	TGGATGTGGT	CAGGTTTGAT	GATGACAACT	CTCCTTCCTT	TATCCAAAAT	1320
	CGCTCAGTTG	CCAAGAAGCA	TCCTAAAAC	TGGGTACATT	ACATTGCTGC	TGAAGAGGAG	1380
	GACTGGGACT	ATGCTCCCTT	AGTCCTCGCC	CCCGATGACA	GAAGTTATAA	AAGTCAATAT	1440
	TTGAACAATG	GCCCTCAGCG	GATTGGTAGG	AAGTACAAAA	AAGTCCGATT	TATGGCATA	1500
5	ACAGATGAAA	CCTTTAAGAC	TCGTGAAGCT	ATTGAGCATG	AATCAGGAAT	CTTGGGACCT	1560
	TTACTTTATG	GGGAAGTTGG	AGACACACTG	TTGATTATAT	TTAAGAATCA	AGCAAGCAGA	1620
	CCATATAACA	TCTACCCCTCA	CGGAATCACT	GATGTCCGTC	CTTTGTATT	AAGGAGATTA	1680
	CCAAAAGGTG	TAAAACATTT	GAAGGATTTT	CCAATTCTGC	CAGGAGAAAT	ATTCAAATAT	1740
	AAATGGACAG	TGACTGTAGA	AGATGGGCCA	ACTAAATCAG	ATCCTCGGTG	CCTGACCCGC	1800
10	TATTACTCTA	GTTTCGTTAA	TATGGAGAGA	GATCTAGCTT	CAGGACTCAT	TGGCCCTCTC	1860
	CTCATCTGCT	ACAAAGAATC	TGTAGATCAA	AGAGGAAACC	AGATAATGTC	AGACAAGAGG	1920
	AATGTCATCC	TGTTTTCTGT	ATTTGATGAG	AACCGAAGCT	GGTACCTCAC	AGAGAATATA	1980
	CAACGCTTTC	TCCCCAATCC	AGCTGGAGTG	CAGCTTGAGG	ATCCAGAGTT	CCAAGCCTCC	2040
	AACATCATGC	ACAGCATCAA	TGGCTATGTT	TTTGATAGTT	TGCAGTTGTC	AGTTTGTTTG	2100
15	CATGAGGTGG	CATACTGGTA	CATTCTAAGC	ATTGGAGCAC	AGACTGACTT	CCTTTCTGTC	2160
	TTCTTCTCTG	GATATACCTT	CAAACACAAA	ATGGTCTATG	AAGACACACT	CACCCTATTC	2220
	CCATTCTCAG	GAGAAACTGT	CTTCATGTCG	ATGGAAAACC	CAGGTCTATG	GATTCTGGGG	2280
	TGCCACAAC	CAGACTTTCG	GAACAGAGGC	ATGACCGCCT	TACTGAAGGT	TTCTAGTTGT	2340
	GACAAGAACA	CTGGTGATTA	TTACGAGGAC	AGTTATGAAG	ATATTTGAGC	ATACTTGCTG	2400
20	AGTAAAAACA	ATGCCATTGA	ACCAAGAAGC	TTCTCCGAGA	ATTCAAGACA	CCCTAGCACT	2460
	AGGCAAAAGC	AATTTAATGC	CACCACAATT	CCAGAAAATG	ACATAGAGAA	GACTGACCCT	2520
	TGGTTTGCAC	ACAGAACACC	TATGCCTAAA	ATACAAAATG	TCTCCTCTAG	TGATTTGTTG	2580
	ATGCTCTTGC	GACAGAGTCC	TACTCCACAT	GGGCTATCCT	TATCTGATCT	CCAAGAAGCC	2640
	AAATATGAGA	CTTTTTCTGA	TGATCCATCA	CCTGGAGCAA	TAGACAGTAA	TAACAGCCTG	2700
25	TCTGAAATGA	CACACTTCAG	GCCACAGCTC	CATCACAGTG	GGGACATGGT	ATTTACCCCT	2760
	GAGTCAGGCC	TCCAATTAAG	ATTAAATGAG	AAACTGGGGA	CAACTGCAGC	AACAGAGTTG	2820
	AAGAACTTGG	ATTTCAAAGT	TTCTAGTACA	TCAAATAATC	TGATTTCAAC	AATTCATCA	2880
	GACAATTTGG	CAGCAGGTAC	TGATAATACA	AGTTCTTAG	GACCCCAAG	TATGCCAGTT	2940
	CATTATGATA	GTCAATTAGA	TACCACTCTA	TTTGGCAAAA	AGTCATCTCC	CCTTACTGAG	3000
30	TCTGGTGGAC	CTCTGAGCTT	GAGTGAAGAA	AATAATGATT	CAAAGTTGTT	AGAATCAGGT	3060
	TTAATGAATA	GCCAAGAAAG	TTCATGGGGA	AAAAATGTAT	CGTCAACAGA	GAGTGGTAGG	3120
	TTATTTAAAG	GGAAAAGAGC	TCATGGACCT	GCTTTGTTGA	CTAAAGATAA	TGCCTTATTC	3180
	AAAGTTAGCA	TCTCTTTGTT	AAAGACAAAC	AAACTTCCA	ATAATTGAGC	AACTAATAGA	3240
	AAGACTCACA	TTGATGGCCC	ATCATTATTA	ATTGAGAATA	GTCCATCAGT	CTGGCAAAT	3300
35	ATATTAGAAA	GTGACACTGA	GTTTAAAAAA	GTGACACCTT	TGATTCATGA	CAGAATGCTT	3360
	ATGGACAAAA	ATGCTACAGC	TTTGAGGCTA	AATCATATGT	CAAATAAAAC	TACTTCATCA	3420
	AAAAACATGG	AAATGGTCCA	ACAGAAAAAA	GAGGGCCCCA	TTCCACCAGA	TGCACAAAAT	3480
	CCAGATATGT	CGTTCTTTAA	GATGCTATTC	TTGCCAGAAT	CAGCAAGGTG	GATACAAAGG	3540
	ACTCATGGAA	AGAACTCTCT	GAACCTCTGG	CAAGGCCCCA	GTCCAAAGCA	ATTAGTATCC	3600
40	TTAGGACCAG	AAAAATCTGT	GGAAGGTCAG	AATTTCTTGT	CTGAGAAAAA	CAAAGTGGTA	3660
	GTAGGAAAGG	GTGAATTTAC	AAAGGACGTA	GGAATCAAAG	AGATGGTTTT	TCCAAGCAGC	3720
	AGAAACCTAT	TTCTTACTAA	CTTGATAAT	TTACATGAAA	ATAATACACA	CAATCAAGAA	3780
	AAAAAAATTC	AGGAAGAAAT	AGAAAAGAAG	GAAACATTAA	TCCAAGAGAA	TGTAGTTTTG	3840
	CCTCAGATAC	ATACAGTGAC	TGGCACTAAG	AATTTTCATGA	AGAACCTTTT	CTTACTGAGC	3900
45	ACTAGGCAAA	ATGTAGAAGG	TTCATATGAG	GGGGCATATG	CTCCAGTACT	TCAAGATTTT	3960
	AGGTCATTAA	ATGATTCAAC	AAATAGAACA	AAGAAACACA	CAGCTCATTT	CTCAAAAAAA	4020
	GGGGAGGAAG	AAAACCTGGA	AGGCTTGGA	AATCAAACCA	AGCAAATTGT	AGAGAAATAT	4080

SUBSTITUTE SHEET (RULE 26)

	GCATGCACCA	CAAGGATATC	TCCTAATACA	AGCCAGCAGA	ATTTTGTGAC	GCAACGTAGT	4140
	AAGAGAGCTT	TGAAACAATT	CAGACTCCCA	CTAGAAGAAA	CAGAACTTGA	AAAAAGGATA	4200
	ATTGTGGATG	ACACCTCAAC	CCAGTGGTCC	AAAAACATGA	AACATTTGAC	CCCGAGCACC	4260
	CTCACACAGA	TAGACTACAA	TGAGAAGGAG	AAAGGGGCCA	TTACTCAGTC	TCCCTTATCA	4320
5	GATTGCCTTA	CGAGGAGTCA	TAGCATCCCT	CAAGCAAATA	GATCTCCATT	ACCCATTGCA	4380
	AAGGTATCAT	CATTTCCATC	TATTAGACCT	ATATATCTGA	CCAGGGTCCT	ATTCCAAGAC	4440
	AACTCTTCTC	ATCTTCCAGC	AGCATCTTAT	AGAAAGAAAG	ATTCTGGGGT	CCAAGAAAGC	4500
	AGTCATTTCT	TACAAGGAGC	CAAAAAAAT	AACCTTTCTT	TAGCCATTCT	AACCTTGGAG	4560
	ATGACTGGTG	ATCAAAGAGA	GGTTGGCTCC	CTGGGGACAA	GTGCCACAAA	TTCAGTCACA	4620
10	TACAAGAAAG	TTGAGAACAC	TGTTCTCCCG	AAACCAGACT	TGCCCAAAAC	ATCTGGCAAA	4680
	GTTGAATTGC	TTCCAAAAGT	TCACATTTAT	CAGAAGGACC	TATTCCCTAC	GGAAACTAGC	4740
	AATGGGTCTC	CTGGCCATCT	GGATCTCGTG	GAAGGGAGCC	TTCTTCAGGG	AACAGAGGGA	4800
	GCGATTAAGT	GGAATGAAGC	AAACAGACCT	GGAAAAGTTC	CCTTTCTGAG	AGTAGCAACA	4860
	GAAAGCTCTG	CAAAGACTCC	CTCCAAGCTA	TTGGATCCTC	TTGCTTGGGA	TAACCACTAT	4920
15	GGTACTCAGA	TACCAAAAGA	AGAGTGGAAA	TCCCAAGAGA	AGTCACCAGA	AAAAACAGCT	4980
	TTTAAGAAAA	AGGATACCAT	TTTGTCCCTG	AACGCTTG TG	AAAGCAATCA	TGCAATAGCA	5040
	GCAATAAATG	AGGGACAAAA	TAAGCCCGAA	ATAGAAGTCA	CCTGGGCAAA	GCAAGGTAGG	5100
	ACTGAAAGGC	TGTGCTCTCA	AAACCCACCA	GTCTTGAAAC	GCCATCAACG	GGAAATAACT	5160
	CGTACTACTC	TTCAAGTCAGA	TCAAGAGGAA	ATTGACTATG	ATGATACCAT	ATCAGTTGAA	5220
20	ATGAAGAAGG	AAGATTTTGA	CATTTATGAT	GAGGATGAAA	ATCAGAGCCC	CCGCAGCTTT	5280
	CAAAGAAAA	CACGACACTA	TTTTATTGCT	GCAGTGGAGA	GGCTCTGGGA	TTATGGGATG	5340
	AGTAGCTCCC	CACATGTTCT	AAGAAACAGG	GCTCAGAGTG	GCAGTGTCCC	TCAGTTCAAG	5400
	AAAGTTGTTT	TCCAGGAATT	TACTGATGGC	TCCTTTACTC	AGCCCTTATA	CCGTGGAGAA	5460
	CTAAATGAAC	ATTTGGGACT	CCTGGGGCCA	TATATAAGAG	CAGAAGTTGA	AGATAATATC	5520
25	ATGGTAACTT	TCAGAAATCA	GGCCTCTCGT	CCCTATTCTT	TCTATTCTAG	CCTTATTTCT	5580
	TATGAGGAAG	ATCAGAGGCA	AGGAGCAGAA	CCTAGAAAAA	ACTTTGTCAA	GCCTAATGAA	5640
	ACCAAAACTT	ACTTTTGGAA	AGTGCAACAT	CATATGGCAC	CCACTAAAGA	TGAGTTTGAC	5700
	TGCAAAGCCT	GGGCTTATTT	CTCTGATGTT	GACCTGGAAA	AAGATGTGCA	CTCAGGCCTG	5760
	ATTGGACCCC	TTCTGGTCTG	CCACACTAAC	ACACTGAACC	CTGCTCATGG	GAGACAAGTG	5820
30	ACAGTACAGG	AATTTGCTCT	GTFTTTTACC	ATCTTTGATG	AGACCAAAAG	CTGGTACTTC	5880
	ACTGAAAATA	TGGAAAGAAA	CTGCAGGGCT	CCCTGCAATA	TCCAGATGGA	AGATCCCACT	5940
	TTTAAAGAGA	ATTATCGCTT	CCATGCAATC	AATGGCTACA	TAATGGATAC	ACTACCTGGC	6000
	TTAGTAATGG	CTCAGGATCA	AAGGATTGGA	TGGTATCTGC	TCAGCATGGG	CAGCAATGAA	6060
	AACATCCATT	CTATTCATTT	CAGTGGACAT	GTGTTCACTG	TACGAAAAAA	AGAGGAGTAT	6120
35	AAAATGGCAC	TGTACAATCT	CTATCCAGGT	GTFTTTTGAGA	CAGTGGAAAT	GTTACCATCC	6180
	AAAGCTGGAA	TTTGGCGGGT	GGAATGCCTT	ATTGGCGAGC	ATCTACATGC	TGGGATGAGC	6240
	ACACTTTTTT	TGGTGTACAG	CAATAAGTGT	CAGACTCCCC	TGGGAATGGC	TTCTGGACAC	6300
	ATTAGAGATT	TTCAAGATTAC	AGCTTCAGGA	CAATATGGAC	AGTGGGCCCC	AAAGCTGGCC	6360
	AGACTTCATT	ATTCGGGATC	AATCAATGCC	TGGAGCACCA	AGGAGCCCTT	TTCTTGGATC	6420
40	AAGGTGGATC	TGTTGGCACC	AATGATTATT	CACGGCATCA	AGACCCAGGG	TGCCCGTCAG	6480
	AAGTTCTCCA	GCCTCTACAT	CTCTCAGTTT	ATCATCATGT	ATAGTCTTGA	TGGGAAGAAG	6540
	TGGCAGACTT	ATCGAGGAAA	TTCCACTGGA	ACCTTAATGG	TCTTCTTTGG	CAATGTGGAT	6600
	TCATCTGGGA	TAAAACACAA	TATTTTTAAC	CCTCCAATTA	TTGCTCGATA	CATCCGTTTG	6660
	CACCCAACTC	ATTATAGCAT	TCGCAGCACT	CTTCGCATGG	AGTTGATGGG	CTGTGATTTA	6720
45	AATAGTTGCA	GCATGCCATT	GGGAATGGAG	AGTAAAGCAA	TATCAGATGC	ACAGATTACT	6780
	GCTTCATCCT	ACTTTACCAA	TATGTTTGCC	ACCTGGTCTC	CTTCAAAGC	TCGACTTCAC	6840
	CTCCAAGGGA	GGAGTAATGC	CTGGAGACCT	CAGGTGAATA	ATCCAAAAGA	GTGGCTGCAA	6900

	GTGGACTTCC	AGAAGACAAT	GAAAGTCACA	GGAGTAACTA	CTCAGGGAGT	AAAATCTCTG	6960
	CTTACCAGCA	TGTATGTGAA	GGAGTTCCTC	ATCTCCAGCA	GTCAAGATGG	CCATCAGTGG	7020
	ACTCTCTTTT	TTTCAAGATGG	CAAAGTAAAG	GTTTTTTCAGG	GAAATCAAGA	CTCCTTCACA	7080
	CCTGTGGTGA	ACTCTCTAGA	CCCACCGTTA	CTGACTCGCT	ACCTTCGAAT	TCACCCCCAG	7140
5	AGTTGGGTGC	ACCAGATTGC	CCTGAGGATG	GAGGTTCTGG	GCTGCGAGGC	ACAGGACCTC	7200
	TACTGAGGGT	GGCCACTGCA	GCACCTGCCA	CTGCCGTCAC	CTCTCCCTCC	TCAGCTCCAG	7260
	GGCAGTGTCC	CTCCCTGGCT	TGCCCTTCTAC	CTTTGTGCTA	AATCCTAGCA	GACACTGCCT	7320
	TGAAGCCTCC	TGAATTAACT	ATCATCAGTC	CTGCATTTCT	TTGGTGGGGG	GCCAGGAGGG	7380
	TGCATCCAAT	TTAACTTAAC	TCTTACCTAT	TTTCTGCAGC	TGCTCCCAGA	TTACTCCTTC	7440
10	CTTCCAATAT	AACTAGGCAA	AAAGAAGTGA	GGAGAAACCT	GCATGAAAGC	ATTCTTCCCT	7500
	GAAAAGTTAG	GCCTCTCAGA	GTCACCACTT	CCTCTGTTGT	AGAAAAACTA	TGTGATGAAA	7560
	CTTTGAAAAA	GATATTTATG	ATGTTAACAT	TTCAAGTTAA	GCCTCATACG	TTTAAAATAA	7620
	AACTCTCAGT	TGTTTATTAT	CCTGATCAAG	CATGGAACAA	AGCATGTTTC	AGGATCAGAT	7680
	CAATACAATC	TTGGAGTCAA	AAGGCAAATC	ATTTGGACAA	TCTGCAAAAT	GGAGAGAATA	7740
15	CAATAACTAC	TACAGTAAAG	TCTGTTTCTG	CTTCCCTTACA	CATAGATATA	ATTATGTTAT	7800
	TTAGTCATTA	TGAGGGGCAC	ATTCTTATCT	CCAAAACCTAG	CATTCTTAAA	CTGAGAATTA	7860
	TAGATGGGGT	TCAAGAATCC	CTAAGTCCCC	TGAAATTATA	TAAGGCATTTC	TGTATAAATG	7920
	CAAATGTGCA	TTTTTCTGAC	GAGTGTCCAT	AGATATAAAG	CCATTGGTCT	TAATTCTGAC	7980
	CAATAAAAAA	ATAAGTCAGG	AGGATGCAAT	TGTTGAAAGC	TTTGAAATAA	AATAACATGT	8040
20	CTTCTTGAAA	TTTGTGATGG	CCAAGAAAGA	AAATGATGAT	GACATTAGGC	TTCTAAAGGA	8100
	CATACATTTA	ATATTTCTGT	GGAAATATGA	GGAAATCCA	TGGTTATCTG	AGATAGGAGA	8160
	TACAAACTTT	GTAATTCTAA	TAATGCACTC	AGTTTACTCT	CTCCCTCTAC	TAATTTCTCTG	8220
	CTGAAAATAA	CACAACAAAA	ATGTAACAGG	GGAAATTATA	TACCGTGACT	GAAAACCTAGA	8280
	GTCCTACTTA	CATAGTTGAA	ATATCAAGGA	GGTCAGAAGA	AAATTGGACT	GGTGAAAACA	8340
25	GAAAAAACAC	TCCAGTCTGC	CATATCACCA	CACAATAGGA	TCCCCCTTCT	TGCCCTCCAC	8400
	CCCCATAAGA	TTGTGAAGGG	TTTACTGCTC	CTTCCATCTG	CCTGCACCCC	TTCACTATGA	8460
	CTACACAGAA	CTCTCCTGAT	AGTAAAGGGG	GCTGGAGGCA	AGGATAAGTT	ATAGAGCAGT	8520
	TGGAGGAAGC	ATCCAAAGAC	TGCAACCCAG	GGCAAATGGA	AAACAGGAGA	TCCTAATATG	8580
	AAAGAAAAAT	GGATCCCAAT	CTGAGAAAAG	GCAAAAGAAT	GGCTACTTTT	TTCTATGCTG	8640
30	GAGTATTTTC	TAATAATCCT	GCTTGACCCCT	TATCTGACCT	CTTTGGAAAC	TATAACATAG	8700
	CTGTCACAGT	ATAGTCACAA	TCCACAAATG	ATGCAGGTGC	AAATGGTTTA	TAGCCCTGTG	8760
	AAGTTCTTAA	AGTTTAGAGG	CTAACTTACA	GAAATGAATA	AGTTGTTTGT	TTTTATAGCC	8820
	CGGTAGAGGA	GTTAACCCCA	AAGGTGATAT	GGTTTTATTT	CCTGTTATGT	TTAACTTGAT	8880
	AATCTTATTT	TGGCATTCTT	TTCCCATTTA	CTATATACAT	CTCTATTTCT	CAATGTTTCA	8940
35	TGGAAGTAGC	TCTTTTATTT	TCCGTCTGGT	TTCTTCAGTA	ATGAGTTAAA	TAAAACATTG	9000
	ACACATACA						9009

(2) INFORMATION FOR SEQ ID NO:16:

- 40 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 8575 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

SUBSTITUTE SHEET (RULE 26)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

	GCAGCTGAGA	GCATGGCCTA	GGGTGGGCGG	CACCATTGTC	CAGCAGCTGA	GTTTCCCAGG	60
5	GACCTTGGAG	ATAGCCGCAG	CCCTCATTTG	CAGGGGAAGA	TGATTCCCTGC	CAGATTTGCC	120
	GGGGTGCTGC	TTGCTCTGGC	CCTCATTTTG	CCAGGGACCC	TTTGTGCAGA	AGGAACTCGC	180
	GGCAGGTCAT	CCACGGCCCG	ATGCAGCCTT	TTCGGAAGTG	ACTTCGTCAA	CACCTTTGAT	240
	GGGAGCATGT	ACAGCTTTGC	GGGATACTGC	AGTTACCTCC	TGGCAGGGGG	CTGCCAGAAA	300
	CGCTCCTTCT	CGATTATTGG	GGACTTCCAG	AATGGCAAGA	GAGTGAGCCT	CTCCGTGTAT	360
10	CTTGGGGGAAT	TTTTTGACAT	CCATTGTGTT	GTCAATGGTA	CCGTGACACA	GGGGGACCAA	420
	AGAGTCTCCA	TGCCCTATGC	CTCCAAAGGG	CTGTATCTAG	AAACTGAGGC	TGGGTACTAC	480
	AAGCTGTCCG	GTGAGGCCTA	TGGCTTTGTG	GCCAGGATCG	ATGGCAGCGG	CAACTTTCAA	540
	GTCTTGCTGT	CAGACAGATA	CTTCAACAAG	ACCTGCGGGC	TGTGTGGCAA	CTTTAACATC	600
	TTTGCTGAAG	ATGACTTTAT	GACCCAAGAA	GGGACCTTGA	CCTCGGACCC	TTATGACTTT	660
15	GCCAACTCAT	GGGCTCTGAG	CAGTGGAGAA	CAGTGGTGTG	AACGGGCATC	TCCTCCCAGC	720
	AGCTCATGCA	ACATCTCCTC	TGGGGAAATG	CAGAAGGGCC	TGTGGGAGCA	GTGCCAGCTT	780
	CTGAAGAGCA	CCTCGGTGTT	TGCCCGCTGC	CACCTCTGCG	TGGACCCCGA	GCCTTTTGTG	840
	GCCCTGTGTG	AGAAGACTTT	GTGTGAGTGT	GCTGGGGGGC	TGGAGTGGCG	CTGCCCTGCC	900
	CTCCTGGAGT	ACGCCCGGAC	CTGTGCCCAG	GAGGGAATGG	TGCTGTACCG	CTGGACCGAC	960
20	CACAGCGCGT	GCAGCCCACT	GTGCCCTGCT	GGTATGGAGT	ATAGGCAGTG	TGTGTCCCTT	1020
	TGCGCCAGGA	CCTGCCAGAG	CCTGCACATC	AATGAAATGT	GTCAGGAGCG	ATGCGTGGAT	1080
	GGCTGCAGCT	GCCCTGAGGG	ACAGCTCCTG	GATGAAGGCC	TCTGCGTGGA	GAGCACCGAG	1140
	TGTCCCTGCG	TGCATTCCGG	AAAGCGCTAC	CCTCCCGGCA	CCTCCCTCTC	TCGAGACTGC	1200
	AACACCTGCA	TTTGCCGAAA	CAGCCAGTGG	ATCTGCAGCA	ATGAAGAATG	TCCAGGGGAG	1260
25	TGCCTTGTCA	CAGGTCAATC	ACACTTCAAG	AGCTTTGACA	ACAGATACTT	CACCTTCAGT	1320
	GGGATCTGCC	AGTACCTGCT	GGCCCGGGAT	TGCCAGGACC	ACTCCTTCTC	CATTGTCAAT	1380
	GAGACTGTCC	AGTGTGCTGA	TGACCGCGAC	GCTGTGTGCA	CCCGCTCCGT	CACCGTCCGG	1440
	CTGCCTGGCC	TGCACAACAG	CCTTGTGAAA	CTGAAGCATG	GGGCAGGAGT	TGCCATGGAT	1500
	GGCCAGGACG	TCCAGCTCCC	CCTCCTGAAA	GGTGACCTCC	GCATCCAGCA	TACAGTGACG	1560
30	GCCTCCGTGC	GCCTCAGCTA	CGGGGAGGAC	CTGCAGATGG	ACTGGGATGG	CCGCGGGAGG	1620
	CTGCTGGTGA	AGCTGTCCCC	CGTCTATGCC	GGGAAGACCT	GCGGCTGTG	TGGGAATTAC	1680
	AATGGCAACC	AGGGCGACGA	CTTCTTACC	CCCTCTGGGC	TGGCGGAGCC	CCGGGTGGAG	1740
	GACTTCGGGA	ACGCTTGGAA	GCTGCACGGG	GACTGCCAGG	ACCTGCAGAA	GCAGCACAGC	1800
	GATCCCTGCG	CCCTCAACCC	GCGCATGACC	AGGTTCTCCG	AGGAGGCGTG	CGCGGTCTTG	1860
35	ACGTCCCCCA	CATTTCGAGG	CTGCCATCGT	GCGTTCAGCC	CGCTGCCCTA	CCTGCGGAAC	1920
	TGCCGCTACG	ACGTGTGCTC	CTGCTCGGAC	GGCCGCGAGT	GCCTGTGCGG	CGCCCTGGCC	1980
	AGCTATGCCG	CGGCTTGGCG	GGGGAGAGGC	GTGCGCGTCG	CGTGGCGCGA	GCCAGGCCGC	2040
	TGTGAGCTGA	ACTGCCCCGA	AGGCCAGGTG	TACCTGCAGT	GCGGGACCCC	CTGCAACCTG	2100
	ACCTGCCGCT	CTCTCTCTTA	CCCGGATGAG	GAATGCAATG	AGGCCTGCCT	GGAGGGCTGC	2160
40	TTCTGCCCCC	CAGGGCTCTA	CATGGATGAG	AGGGGGGACT	GCGTGCCCAA	GGCCCACTGC	2220
	CCCTGTTACT	ATGACGGTGA	GATCTTCCAG	CCAGAAGACA	TCTTCTCAGA	CCATCACACC	2280
	ATGTGCTACT	GTGAGGATGG	CTTCATGCAC	TGTACCATGA	GTGGAGTCCC	CGGAAGCTTG	2340
	CTGCCCTGACG	CTGTCTTCAG	CAGTCCCCCTG	TCTCATCGCA	GCAAAAGGAG	CCTATCTCTGT	2400
	CGGCCCCCCA	TGGTCAAGCT	GGTGTGTCCC	GCTGACAACC	TGCGGGCTGA	AGGGCTCGAG	2460
45	TGTACCAAAA	CGTGCCAGAA	CTATGACCTG	GAGTGCATGA	GCATGGGCTG	TGTCTCTGGC	2520
	TGCCTCTGCC	CCCCGGGCAT	GGTCCGGCAT	GAGAACAGAT	GTGTGGCCCT	GGAAAGGTGT	2580
	CCCTGCTTCC	ATCAGGGCAA	GGAGTATGCC	CCTGGAGAAA	CAGTGAAGAT	TGGCTGCAAC	2640

SUBSTITUTE SHEET (RULE 26)

	ACTTGTGTCT	GTCCGGGACCG	GAAGTGGAAAC	TGCACAGACC	ATGTGTGTGA	TGCCACGTGC	2700
	TCCACGATCG	GCATGGCCCA	CTACCTCACC	TTCGACGGGC	TCAAATACCT	GTTCCCCGGG	2760
	GAGTGCCAGT	ACGTTCTGGT	GCAGGATTAC	TGCGGCAGTA	ACCCTGGGAC	CTTTCGGATC	2820
	CTAGTGGGGA	ATAAGGGATG	CAGCCACCCC	TCAGTGAAAT	GCAAGAAACG	GGTCACCATC	2880
5	CTGGTGGAGG	GAGGAGAGAT	TGAGCTGTTT	GACGGGGAGG	TGAATGTGAA	GAGGCCCCATG	2940
	AAGGATGAGA	CTCACTTTGA	GGTGGTGGAG	TCTGGCCGGT	ACATCATTCT	GCTGCTGGGC	3000
	AAAGCCCTCT	CCGTGGTCTG	GGACCGCCAC	CTGAGCATCT	CCGTGGTCTT	GAAGCAGACA	3060
	TACCAGGAGA	AAGTGTGTGG	CCTGTGTGGG	AATTTTGTATG	GCATCCAGAA	CAATGACCTC	3120
	ACCAGCAGCA	ACCTCCAAGT	GGAGGAAGAC	CCTGTGGACT	TTGGGAACTC	CTGGAAAGTG	3180
10	AGCTCGCAGT	GTGCTGACAC	CAGAAAAGTG	CCTCTGGACT	CATCCCCTGC	CACCTGCCAT	3240
	AACAACATCA	TGAAGCAGAC	GATGGTGGAT	TCCTCCTGTA	GAATCCTTAC	CAGTGACGTC	3300
	TTCCAGGACT	GCAACAAGCT	GGTGGACCCC	GAGCCATATC	TGGATGTCTG	CATTTACGAC	3360
	ACCTGCTCCT	GTGAGTCCAT	TGGGGACTGC	GCCTGCTTCT	GCGACACCAT	TGCTGCCTAT	3420
	GCCCCAGTGT	GTGCCCAGCA	TGGCAAGGTG	GTGACCTGGA	GGACGGCCAC	ATTGTGCCCC	3480
15	CAGAGCTGCG	AGGAGAGGAA	TCTCCGGGAG	AACGGGTATG	AGTGTGAGTG	GCGCTATAAC	3540
	AGCTGTGCAC	CTGCCTGTCA	AGTCACGTGT	CAGCACCCCTG	AGCCACTGGC	CTGCCCTGTG	3600
	CAGTGTGTGG	AGGGCTGCCA	TGCCCCACTGC	CCTCCAGGGA	AAATCCTGGA	TGAGCTTTTG	3660
	CAGACCTGCG	TTGACCCTGA	AGACTGTCCA	GTGTGTGAGG	TGGCTGGCCG	GCGTTTTGCC	3720
	TCAGGAAAGA	AAGTCACCTT	GAATCCAGT	GACCCTGAGC	ACTGCCAGAT	TTGCCACTGT	3780
20	GATGTTGTCA	ACCTCACCTG	TGAAGCCTGC	CAGGAGCCGG	GAGGCCTGGT	GGTGCCTCCC	3840
	ACAGATGCCC	CGGTGAGCCC	CACCACTCTG	TATGTGGAGG	ACATCTCGGA	ACCGCCGTTG	3900
	CACGATTTCT	ACTGCAGCAG	GCTACTGGAC	CTGGTCTTCC	TGCTGGATGG	CTCCTCCAGG	3960
	CTGTCCGAGG	CTGAGTTTGA	AGTGCTGAAG	GCTTTTGTGG	TGGACATGAT	GGAGCGGCTG	4020
	CGCATCTCCC	AGAAGTGGGT	CCGCGTGGCC	GTGGTGGAGT	ACCACGACGG	CTCCACGCC	4080
25	TACATCGGGC	TCAAGGACCG	GAAGCGACCG	TCAGAGCTGC	GGCGCATTGC	CAGCCAGGTG	4140
	AAGTATGCGG	GCAGCCAGGT	GGCCTCCACC	AGCGAGGTCT	TGAAATACAC	ACTGTTCCAA	4200
	ATCTTCAGCA	AGATCGACCG	CCCTGAAGCC	TCCCGCATCG	CCCTGCTCCT	GATGGCCAGC	4260
	CAGGAGCCCC	AACGGATGTC	CCGGAACCTT	GTCCGCTACG	TCCAGGGCCT	GAAGAAGAAG	4320
	AAGGTCATTG	TGATCCCGGT	GGGCATTGGG	CCCCATGCCA	ACCTCAAGCA	GATCCGCCTC	4380
30	ATCGAGAAGC	AGGCCCCCTGA	GAACAAGGCC	TTCGTGCTGA	GCAGTGTGGA	TGAGCTGGAG	4440
	CAGCAAAGGG	ACGAGATCGT	TAGCTACCTC	TGTGACCTTG	CCCCTGAAGC	CCCTCCTCCT	4500
	ACTCTGCCCC	CCCACATGGC	ACAAGTCACT	GTGGGCCCCG	GGCTCTTGGG	GGTTTCGACC	4560
	CTGGGGCCCA	AGAGGAACTC	CATGGTTCTG	GATGTGGCGT	TCGTCTTGGA	AGGATCGGAC	4620
	AAAATTGGTG	AAGCCGACTT	CAACAGGAGC	AAGGAGTTCA	TGGAGGAGGT	GATTTCAGCG	4680
35	ATGGATGTGG	GCCAGGACAG	CATCCACGTC	ACGGTGCTGC	AGTACTCCTA	CATGGTGACC	4740
	GTGGAGTACC	CCTTCAGCGA	GGCACAGTCC	AAAGGGGACA	TCCTGCAGCG	GGTGCAGAG	4800
	ATCCGCTACC	AGGGCGGCAA	CAGGACCAAC	ACTGGGCTGG	CCCTGCGGTA	CCTCTCTGAC	4860
	CACAGCTTCT	TGGTCAGCCA	GGGTGACCGG	GAGCAGGCGC	CCAACCTGGT	CTACATGGTC	4920
	ACCGGAAATC	CTGCCTCTGA	TGAGATCAAG	AGGCTGCCTG	GAGACATCCA	GGTGGTGCCC	4980
40	ATTGGAGTGG	GCCCTAATGC	CAACGTGCAG	GAGCTGGAGA	GGATTGGCTG	GCCCAATGCC	5040
	CCTATCCTCA	TCCAGGACTT	TGAGACGCTC	CCCCGAGAGG	CTCCTGACCT	GGTGCTGCAG	5100
	AGGTGCTGCT	CCGGAGAGGG	GCTGCAGATC	CCCACCTCTT	CCCCTGCACC	TGACTGCAGC	5160
	CAGCCCCTGG	ACGTGATCCT	TCTCCTGGAT	GGCTCCTCCA	GTTTCCCAGC	TTCTTATTTT	5220
	GATGAAATGA	AGAGTTTCGC	CAAGGCTTTC	ATTTCAAAAG	CCAATATAGG	GCCTCGTCTC	5280
45	ACTCAGGTGT	CAGTGCTGCA	GTATGGAAGC	ATCACCACCA	TTGACGTGCC	ATGGAACGTG	5340
	GTCCCGGAGA	AAGCCCATTT	GCTGAGCCTT	GTGGACGTCA	TGCAGCGGGA	GGGAGGCCCC	5400
	AGCCAAATCG	GGGATGCCTT	GGGCTTTGCT	GTGCGATACT	TGACTTCAGA	AATGCATGGT	5460

SUBSTITUTE SHEET (RULE 26)

	GCCAGGCCGG	GAGCCTCAAA	GGCGGTGGTC	ATCCTGGTCA	CGGACGTCTC	TGTGGATTCA	5520
	GTGGATGCAG	CAGCTGATGC	CGCCAGGTCC	AACAGAGTGA	CAGTGTTCCTC	TATTGGAATT	5580
	GGAGATCGCT	ACGATGCAGC	CCAGCTACGG	ATCTTGGCAG	GCCCAGCAGG	CGACTCCAAC	5640
	GTGGTGAAGC	TCCAGCGAAT	CGAAGACCTC	CCTACCATGG	TCACCTTGGG	CAATTCCCTC	5700
5	CTCCACAAAC	TGTGCTCTGG	ATTTGTTAGG	ATTTGTCATGG	ATGAGGATGG	GAATGAGAAG	5760
	AGGCCCCGGG	ACGTCTGGAC	CTTGCCAGAC	CAGTGCCACA	CCGTGACTTG	CCAGCCAGAT	5820
	GGCCAGACCT	TGCTGAAGAC	TCATCGGGTC	AACTGTGACC	GGGGGCTGAG	GCCTTCGTGC	5880
	CCTAACAGCC	AGTCCCCTGT	TAAAGTGGAA	GAGACCTGTG	GCTGCGCTG	GACCTGCCCC	5940
	TGCGTGTGCA	CAGGCAGCTC	CACTCGGCAC	ATCGTGACCT	TTGATGGGCA	GAATTTCAAG	6000
10	CTGACTGGCA	GCTGTTCTTA	TGTCCTATTT	CAAAACAAGG	AGCAGGACCT	GGAGGTGATT	6060
	CTCCATAATG	GTGCCTGCAG	CCCTGGAGCA	AGGCAGGGCT	GCATGAAATC	CATCGAGGTG	6120
	AAGCACAGTG	CCCTCTCCGT	CGAGCTGCAC	AGTGACATGG	AGGTGACGGT	GAATGGGAGA	6180
	CTGGTCTCTG	TTCTTTACGT	GGGTGGGAAC	ATGGAAGTCA	ACGTTTATGG	TGCCATCATG	6240
	CATGAGGTCA	GATTCAATCA	CCTTGGTCAC	ATCTTCACAT	TCACTCCACA	AAACAATGAG	6300
15	TTCCAACCTG	AGCTCAGCCC	CAAGACTTTT	GCTTCAAAGA	CGTATGGTCT	GTGTGGGATC	6360
	TGTGATGAGA	ACGGAGCCAA	TGACTTCATG	CTGAGGGATG	GCACAGTCAC	CACAGACTGG	6420
	AAAACACTTG	TTCAAGGAATG	GACTGTGCAG	CGGCCAGGGC	AGACGTGCCA	GCCCATCCTG	6480
	GAGGAGCAGT	GTCTTGTCCC	CGACAGCTCC	CACTGCCAGG	TCCTCCTCTT	ACCACTGTTT	6540
	GCTGAATGCC	ACAAGGTCCCT	GGCTCCAGCC	ACATTCTATG	CCATCTGCCA	GCAGGACAGT	6600
20	TGCCACCAGG	AGCAAGTGTG	TGAGGTGATC	GCCTCTTATG	CCCACCTCTG	TCCGACCAAC	6660
	GGGGTCTGCG	TTGACTGGAG	GACACCTGAT	TTCTGTGCTA	TGTCATGCCC	ACCATCTCTG	6720
	GTCTACAACC	ACTGTGAGCA	TGGCTGTCCC	CGGCACTGTG	ATGGCAACGT	GAGCTCCTGT	6780
	GGGGACCATC	CCTCCGAAGG	CTGTTTCTGC	CCTCCAGATA	AAGTCATGTT	GGAAGGCAGC	6840
	TGTGTCCCTG	AAGAGGCCTG	CACTCAGTGC	ATTGGTGAGG	ATGGAGTCCA	GCACCAGTTC	6900
25	CTGGAAGCCT	GGGTCCCGGA	CCACCAGCCC	TGTCAGATCT	GCACATGCCT	CAGCGGGCGG	6960
	AAGGTCAACT	GCACAACGCA	GCCCTGCCCC	ACGGCCAAAG	CTCCCACGTG	TGGCCTGTGT	7020
	GAAGTAGCCC	GCCTCCGCCA	GAATGCAGAC	CAGTGCTGCC	CCGAGTATGA	GTGTGTGTGT	7080
	GACCCAGTGA	GCTGTGACCT	GCCCCCAGTG	CCTCACTGTG	AACGTGGCCT	CCAGCCCACA	7140
	CTGACCAACC	CTGGCGAGTG	CAGACCCAAC	TTCACCTGCG	CCTGCAGGAA	GGAGGAGTGC	7200
30	AAAAGAGTGT	CCCCACCCCTC	CTGCCCCCGG	CACCGTTTGC	CCACCCTTCG	GAAGACCCAG	7260
	TGCTGTGATG	AGTATGAGTG	TGCCTGCAAC	TGTGTCAACT	CCACAGTGAG	CTGTCCCCCT	7320
	GGGTACTTGG	CCTCAACCGC	CACCAATGAC	TGTGGCTGTA	CCACAACCAC	CTGCCTTCCC	7380
	GACAAGGTGT	GTGTCCACCG	AAGCACCATC	TACCTGTGG	GCCAGTTCTG	GGAGGAGGGC	7440
	TGCGATGTGT	GCACCTGCAC	CGACATGGAG	GATGCCGTGA	TGGGCCTCCG	CGTGGCCCAG	7500
35	TGCTCCGAGA	AGCCCTGTGA	GGACAGCTGT	CGGTCCGGCT	TCACTTACGT	TCTGCATGAA	7560
	GGCGAGTGCT	GTGGAAGGTG	CCTGCCATCT	GCCTGTGAGG	TGGTGACTGG	CTCACCGCGG	7620
	GGGGACTCCC	AGTCTTCCTG	GAAGAGTGTG	GGCTCCAGT	GGGCCTCCCC	GGAGAACCCC	7680
	TGCCTCATCA	ATGAGTGTGT	CCGAGTGAAG	GAGGAGGTCT	TTATACAACA	AAGGAACGTC	7740
	TCCTGCCCCC	AGCTGGAGGT	CCCTGTCTGC	CCCTCCGGCT	TTCACTGAG	CTGTAAGACC	7800
40	TCAGCGTGCT	GCCCAAGCTG	TGCTGTGAG	CGCATGGAGG	CCTGCATGCT	CAATGGCACT	7860
	GTCATTGGGC	COGGGAAGAC	TGTGATGATC	GATGTGTGCA	CGACCTGCCG	CTGCATGGTG	7920
	CAGGTGGGGG	TCATCTCTGG	ATTCAAGCTG	GAGTGCAGGA	AGACCACCTG	CAACCCCTGC	7980
	CCCCTGGGTT	ACAAGGAAGA	AAATAACACA	GGTGAATGTT	GTGGGAGATG	TTTGCCTACG	8040
	GCTTGACCA	TTCACTAAG	AGGAGGACAG	ATCATGACAC	TGAAGCGTGA	TGAGACGCTC	8100
45	CAGGATGGCT	GTGATACTCA	CTTCTGCAAG	GTCAATGAGA	GAGGAGAGTA	CTTCTGGGAG	8160
	AAGAGGGTCA	CAGGCTGCCC	ACCCCTTTGAT	GAACACAAGT	GTCTGGCTGA	GGGAGGTAAA	8220
	ATTATGAAAA	TTCCAGGCAC	CTGCTGTGAC	ACATGTGAGG	AGCCTGAGTG	CAACGACATC	8280

ACTGCCAGGC	TGCAGTATGT	CAAGGTGGGA	AGCTGTAAGT	CTGAAGTAGA	GGTGGATATC	8340
CACTACTGCC	AGGGCAAATG	TGCCAGCAAA	GCCATGTACT	CCATTGACAT	CAACGATGTG	8400
CAGGACCAAGT	GCTCCTGCTG	CTCTCOGACA	CGGACGGAGC	CCATGCAGGT	GGCCCTGCAC	8460
TGCACCAATG	GCTCTGTTGT	GTACCATGAG	GTTCTCAATG	CCATGGAGTG	CAAATGCTCC	8520
5 CCCAGGAAGT	GCAGCAAGTG	AGGCTGCTGC	AGCTGCATGG	GTGCCTGCTG	CTGCC	8575

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13815 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

20	CCTCACTCAC	TTGCCCCCTTA	CAGGACTCAG	CTCTTGAAGG	CAATAGCTTTT	ATAGAAAAAA	60
	CGAATAGGAA	GACTTGAAGT	GCTATTTTTT	TTTTTTTTTT	TGTCAAGGCT	GCTGAAGTTT	120
	ATTGGCTTCT	CATCGTACCT	AAGCCTCCTG	GAGCAATAAA	ACTGGGAGAA	ACTTTTACCA	180
	AGATTTTTAT	CCCTGCCTTG	ATATATACTT	TTCTTTCCAA	ATGCTTTGGT	GGGAAGAAGT	240
25	AGAGGACTGT	TATGAAAGAG	AAGATGTTCA	AAAGAAAACA	TTCACAAAAT	GGATAAATGC	300
	ACAATTTTCT	AAGTTTGGAA	AGCAACACAT	AGACAACCTC	TTCAGTGACC	TGCAGGATGG	360
	AAAACGCCTC	CTAGACCTCT	TGGAAGGCCT	TACAGGGCAA	AAACTGCCAA	AAGAAAAGGG	420
	ATCTACAAGA	GTTTCATGCC	TGAACAATGT	CAACAAGGCA	CTGCGGGTCT	TACAGAAAAA	480
	TAATGTTGAT	TTAGTGAATA	TAGGAAGCAC	TGACATAGTG	GATGGAAATC	ATAAACTCAC	540
30	TCTTGGTTTG	ATTTGGAATA	TAATCCTCCA	CTGGCAGGTC	AAAAATGTGA	TGAAAACAT	600
	CATGGCTGGA	TTGCAGCAAA	CCAACAGTGA	AAAGATTCTT	CTGAGCTGGG	TTGACAGTC	660
	AACACGTAAT	TATCCACAGG	TTAACGTCAT	CAACTTCACC	TCTAGCTGGT	CCGACGGGTT	720
	GGCTTTGAAT	GCTCTTATCC	ATAGTCACAG	GCCCAGCCTG	TTTGATTGGA	ATAGTGTGGT	780
	TTCACAGCAC	TCAGCCACCC	AAAGACTGGA	ACATGCCTTC	AACATTGCAA	AATGCCAGTT	840
35	AGGCATAGAA	AAACTTCTTG	ATCCTGAAGA	TGTTGCTACC	ACTTATCCAG	ACAAGAAGTC	900
	CATCTTAATG	TACATCACAT	CACTCTTTCA	AGTTTTGCCA	CAACAAGTGA	GCATTGAAGC	960
	CATTCAAGAA	GTGGAAATGT	TGCCCAGGAC	ATCTTCAAAA	GTAAGTAGAG	AAGAACATTT	1020
	TCAATTACAT	CACCAGATGC	ATTACTCTCA	ACAGATCACA	GTCAGTCTAG	CACAGGGCTA	1080
	TGAACAAACT	TCTTCATCTC	CTAAGCCTCG	ATTCAAGAGT	TATGCCTTCA	CACAGGCTGC	1140
40	TTATGTTGCC	ACCTCTGATT	CCACACAGAG	CCCCTATCCT	TCACAGCATT	TGGAAGCTCC	1200
	CAGAGACAAG	TCACTTGACA	GTTTCATTGAT	GGAGACGGAA	GTAAATCTGG	ATAGTTACCA	1260
	AACTGCTTTA	GAAGAAGTAC	TTTCATGGCT	TCTTTCTGCC	GAGGATACAT	TGCGAGCACA	1320
	AGGAGAGATT	TCAAATGATG	TTGAAGAAGT	GAAAGAACAG	TTTCATGCTC	ATGAGGGATT	1380
	CATGATGGAT	CTGACATCTC	ATCAAGGACT	TGTTGGTAAT	GTTCTACAGT	TAGGAAGTCA	1440
45	ACTAGTTGGA	AAAGGGAAAT	TATCAGAAGA	TGAAGAAGCT	GAAGTGCAAG	AACAAATGAA	1500
	TCTCCTAAAT	TCAAGATGGG	AATGTCTCAG	GGTAGCTAGC	ATGGAAAAAC	AAAGCAAATT	1560
	ACACAAAGTT	CTAATGGATC	TCCAGAATCA	GAAATTAAAA	GAAGTAGATG	ACTGGTTAAC	1620

SUBSTITUTE SHEET (RULE 26)

	AAAACTGAA	GAGAGAACTA	AGAAAATGGA	GGAAGAGCCC	TTTGGACCTG	ATCTTGAAGA	1680
	TCTAAATGC	CAAGTACAAC	AACATAAGGT	GCTTCAAGAA	GATCTAGAAC	AGGAGCAGGT	1740
	CAGGGTCAAC	TCGCTCACTC	ACATGGTAGT	AGTGGTTGAT	GAATCCAGCG	GTGATCATGC	1800
	AACAGCTGCT	TTGGAAGAAC	AACTTAAGGT	ACTGGGAGAT	CGATGGGCAA	ATATCTGCAG	1860
5	ATGGACTGAA	GACCGCTGGA	TTGTTTTACA	AGATATTCTT	CTAAAATGGC	AGCATTTTAC	1920
	TGAAGAACAG	TGCCTTTTTA	GTACATGGCT	TTCAGAAAAA	GAAGATGCAA	TGAAGAACAT	1980
	TCAGACAAGT	GGCTTTTAAAG	ATCAAAATGA	AATGATGTCA	AGTCTTCACA	AAATATCTAC	2040
	TTTTAAAAATA	GATCTAGAAA	AGAAAAAGCC	AACCATGGAA	AAACTAAGTT	CACTCAATCA	2100
	AGATCTACTT	TCGGCACTGA	AAAATAAGTC	AGTGACTCAA	AAGATGGAAA	TCTGGATGGA	2160
10	AAACTTTGCA	CAACGTTGGG	ACAATTTAAC	CCAAAACTT	GAAAAGAGTT	CAGCACAAAT	2220
	TTCACAGGCT	GTCACCACCA	CTCAACCATC	CCTAACACAG	ACAACGTAA	TGGAAACGGT	2280
	AACTATGGTG	ACCACAAGGG	AACAAATCAT	GGTAAACAT	GCCCAAGAGG	AACTTCCACC	2340
	ACCACCTCCT	CAAAAGAAGA	GGCAGATAAC	TGTGGATTCT	GAACTCAGGA	AAAGGTTGGA	2400
	TGTCGATATA	ACTGAACTTC	ACAGTTGGAT	TACTCGTTCA	GAAGCTGTAT	TACAGAGTTC	2460
15	TGAATTTGCA	GTCTATCGAA	AAGAAGGCAA	CATCTCAGAC	TTGCAAGAAA	AAGTCAATGC	2520
	CATAGCACGA	GAAAAAGCAG	AGAAGTTCAG	AAAAGTGCAA	GATGCCAGCA	GATCAGCTCA	2580
	GGCCCTGGTG	GAACAGATGG	CAAATGAGGG	TGTTAATGCT	GAAAGTATCA	GACAAGCTTC	2640
	AGAACAACCTG	AACAGCCGGT	GGACAGAATT	CTGCCAATTG	CTGAGTGAGA	GAGTTAACTG	2700
	GCTAGAGTAT	CAAACCAACA	TCATTACCTT	TTATAATCAG	CTACAACAAT	TGGAACAGAT	2760
20	GACAACTACT	GCCGAAAAC	TGTTGAAAAC	CCAGTCTACC	ACCCTATCAG	AGCCAACAGC	2820
	AATTAAAAGC	CAGTTAAAAA	TTTGTAAGGA	TGAAGTCAAC	AGATTGTCAG	CTCTTCAGCC	2880
	TCAAATTGAG	CAATTAAAAA	TTCAGAGTCT	ACAAGTGAAA	GAAAAGGGAC	AGGGGCCAAT	2940
	GTTCCTGGAT	GCAGACTTTG	TGGCCTTTAC	TAATCATTTT	AACCACATCT	TTGATGGTGT	3000
	GAGGGCCAAA	GAGAAAGAGC	TACAGACAAT	TTTTGACACT	TTACCACCAA	TGCGCTATCA	3060
25	GGAGACAATG	AGTAGCATCA	GGACGTGGAT	CCAGCAGTCA	GAAAGCAAAC	TCTCTGTACC	3120
	TTATCTTAGT	GTTACTGAAT	ATGAAATAAT	GGAGGAGAGA	CTCGGGAAAT	TACAGGCTCT	3180
	GCAAAGTTCT	TTGAAAGAGC	AACAAAATGG	CTTCAACTAT	CTGAGTGACA	CTGTGAAGGA	3240
	GATGGCCAAG	AAAGCACCTT	CAGAAATATG	CCAGAAATAT	CTGTCAGAAT	TTGAAGAGAT	3300
	TGAGGGGCAC	TGGAAGAAAC	TTTCCTCCCA	GTTGGTGGA	AGCTGCCAAA	AGCTAGAAGA	3360
30	ACATATGAAT	AAACTTCGAA	AATTTTCAGAA	TCACATAAAA	ACCTTACAGA	AATGGATGGC	3420
	TGAAGTTGAT	GTTTTCTCTGA	AAGAGGAATG	GCCTGCCCTG	GGGGATGCTG	AAATCCTGAA	3480
	AAAACAGCTC	AAACAATGCA	GACTTTTAGT	TGGTGATATT	CAAACAATTC	AGCCCAGTTT	3540
	AAATAGTGTT	AATGAAGGTG	GGCAGAAGAT	AAAGAGTGAA	GCTGAACTTG	AGTTTGCATC	3600
	CAGACTGGAG	ACAGAACTTA	GAGAGCTTAA	CACTCAGTGG	GATCACATAT	GCCGCCAGGT	3660
35	CTACACCAGA	AAGGAAGCCT	TAAAGGCAGG	TTTGGATAAA	ACCGTAAGCC	TCCAAAAAGA	3720
	TCTATCAGAG	ATGCATGAGT	GGATGACACA	AGCTGAAGAA	GAATATCTAG	AGAGAGATTT	3780
	TGAATATAAA	ACTCCAGATG	AATTACAGAC	TGCTGTTGAA	GAAATGAAGA	GAGCTAAAGA	3840
	AGAGGCACTA	CAAAAAGAAA	CTAAAGTGAA	ACTCCTTACT	GAGACTGTAA	ATAGTGTAAT	3900
	AGCTCACGCT	CCACCCTCAG	CACAAGAGGC	CTTAAAAAAG	GAACCTTGAAA	CTCTGACCAC	3960
40	CAACTACCAA	TGGCTGTGCA	CCAGGCTGAA	TGGAAAATGC	AAAACTTTGG	AAGAAGTTTG	4020
	GGCATGTTGG	CATGAGTTAT	TGTCATATTT	AGAGAAAGCA	AACAAGTGGC	TCAATGAAGT	4080
	AGAATTGAAA	CTTAAACCA	TGGAAAATGT	TCCTGCAGGA	CCTGAGGAAA	TCACTGAAGT	4140
	GCTAGAATCT	CTTGAAAATC	TGATGCATCA	TTCAGAGGAG	AACCCAAATC	AGATTCTGCT	4200
	ATTGGCACAG	ACTCTTACAG	ATGGAGGAGT	CATGGATGAA	CTGATCAATG	AGGAGCTTGA	4260
45	GACGTTTAAT	TCTCGTTGGA	GGGAACTACA	TGAAGAGGCT	GTGAGGAAAC	AAAAGTTGCT	4320
	TGAACAGAGT	ATCCAGTCTG	CCCAGGAAAT	TGAAAAGTCC	TTGCACTTAA	TTCAGGAGTC	4380
	GCTTGAATTC	ATTGACAAGC	AGTTGGCAGC	TTATATCACT	GACAAGGTGG	ATGCAGCTCA	4440

SUBSTITUTE SHEET (RULE 26)

	AATGCCTCAG	GAAGCCCAGA	AAATCCAATC	AGATTGACA	AGTCATGAGA	TAAGTTTAGA	4500
	AGAAATGAAG	AAACATAACC	AGGGGAAGGA	TGCCAACCAA	AGGGTTCTTT	CACAAATTGA	4560
	TGTTGCACAG	AAAAAATTAC	AAGATGTCTC	CATGAAATTT	CGATTATTCC	AAAAACCAGC	4620
	CAATTTTGAA	CAACGTCTAG	AGGAAAGTAA	GATGATTTTA	GATGAAGTCA	AGATGCATTT	4680
5	GCCTGCATTG	GAAACCAAGA	GTGTTGAACA	GGAAGTAATT	CAGTCACAAC	TAAGTCATTG	4740
	TGTGAACTTG	TATAAAAGCC	TGAGTGAAGT	CAAGTCTGAA	GTGGAAATGG	TGATTAAAAC	4800
	CGGACGTCAA	ATTGTACAGA	AAAAGCAGAC	AGAAAATCCC	AAAGAGCTTG	ATGAACGAGT	4860
	AACAGCTTTG	AAATTGCATT	ACAATGAGTT	GGGTGCGAAG	GTAACAGAGA	GAAAGCAACA	4920
	GTGAGAGAAA	TGCTTGAAGT	TGTCCCGTAA	GATGAGAAAG	GAAATGAATG	TCTTAACAGA	4980
10	ATGGCTGGCA	GCAACAGATA	CAGAATTGAC	GAAGAGATCA	GCAGTTGAAG	GAATGCCAAG	5040
	TAATTTGGAT	TCTGAAGTTG	CCTGGGGAAA	GGCTACTCAA	AAAGAGATTG	AGAAACAGAA	5100
	GGCTCACTTG	AAGAGTGTTA	CAGAATTAGG	AGAGTCTTTG	AAAATGGTGT	TGGGCAAGAA	5160
	AGAAACCTTG	GTAGAAGATA	AACTGAGTCT	TCTGAACAGT	AACTGGATAG	CTGTCACCTC	5220
	CAGAGTAGAA	GAATGGCTAA	ATCTTTTGT	GGAATACCAG	AAACACATGG	AAACCTTTGA	5280
15	TCAGAACATA	GAACAAATCA	CAAAGTGGAT	CATTCATGCA	GATGAACTTT	TAGATGAGTC	5340
	TGAAAAGAAG	AAACCACAAC	AAAAGGAAGA	CATTCTTAAG	CGTTTAAAGG	CTGAAATGAA	5400
	TGACATGCGC	CCAAAGGTGG	ACTCCACACG	TGACCAAGCA	GCAAAATTGA	TGGCAAACCG	5460
	CGGTGACCAC	TGCAGGAAAG	TAGTAGAGCC	CCAAATCTCT	GAGCTCAACC	GTGATTTTGC	5520
	AGCTATTTCT	CACAGAATTA	AGACTGGAAA	GGCCTCCATT	CCTTTGAAGG	AATTGGAGCA	5580
20	GTTTAACTCA	GATATACAAA	AATTGCTTGA	ACCACTGGAG	GCTGAAATTC	AGCAGGGGGT	5640
	GAATCTGAAA	GAGGAAGACT	TCAATAAAGA	TATGAGTGAA	GACAATGAGG	GTACTGTAAA	5700
	TGAATTGTTG	CAAAGAGGAG	ACAACTTACA	ACAAAGAATC	ACAGATGAGA	GAAAGCGAGA	5760
	GGAAATAAAG	ATAAAACAGC	AGCTGTTACA	GACAAAACAT	AATGCTCTCA	AGGATTTGAG	5820
	GTCTCAAAGA	AGAAAAAAGG	CCCTAGAAAT	TTCTCACCAG	TGGTATCAGT	ACAAGAGGCA	5880
25	GGCTGATGAT	CTCCTGAAAT	GCTTGGATGA	AATTGAAAAA	AAATTAGCCA	GCCTACCTGA	5940
	ACCCAGAGAT	GAAAGAAAAT	TAAAGGAAAT	TGATCGTGAA	TTGCAGAAGA	AGAAAGAGGA	6000
	GCTGAATGCA	GTGCGCAGGC	AAGCTGAGGG	CTTGTCTGAG	AATGGGGCCG	CAATGGCAGT	6060
	GGAGCCAACT	CAGATCCAGC	TCAGCAAGCG	CTGGCGGCAA	ATTGAGAGCA	ATTTTGCTCA	6120
	GTTCGAAGA	CTCAACTTTG	CACAAATTCA	CACTCTCCAT	GAAGAAACTA	TGGTAGTGAC	6180
30	GACTGAAGAT	ATGCCTTTGG	ATGTTTCTTA	TGTGCCTTCT	ACTTATTTTGA	CCGAGATCAG	6240
	TCATATCTTA	CAAGCTCTTT	CAGAAGTTGA	TCATCTTCTA	AATACTCCTG	AACTCTGTGC	6300
	TAAAGATTTT	GAAGATCTTT	TTAAGCAAGA	GGAGTCTCTT	AAGAATATAA	AAGACAATTT	6360
	GCAACAAATC	TCAGGTCCGA	TTGATATTAT	TCACAAGAAG	AAGACAGCAG	CCTTGCAAAG	6420
	TGCCACCTCC	ATGGAAAAGG	TGAAAGTACA	GGAAGCCGTG	GCACAGATGG	ATTTCCAGGG	6480
35	GGAAAACTT	CATAGAATGT	ACAAGGAACG	ACAAGGGCGA	TTGACAGAT	CAGTTGAAAA	6540
	ATGGCGACAC	TTTCATTATG	ATATGAAGGT	ATTTAATCAA	TGGCTGAATG	AAGTTGAACA	6600
	GTTTTTCAAA	AAGACACAAA	ATCCTGAAAA	CTGGGAACAT	GCTAAATACA	AATGGTATCT	6660
	TAAGGAACTC	CAGGATGGCA	TTGGGCAGCG	TCAAGCTGTT	GTCAGAACAC	TGAATGCAAC	6720
	TGGGGAAGAA	ATAATTCAAC	AGTCTTCAAA	AACAGATGTC	AATATTCTAC	AAGAAAAATT	6780
40	AGGAAGCTTG	AGTCTGCGGT	GGCAGGACAT	CTGCAAAGAG	CTGGCAGAAA	GGAGAAAGAG	6840
	GATTGAAGAA	CAAAGAATG	TCTTGTGAGA	ATTTCAAAGA	GATTTAAATG	AATTTGTTTT	6900
	GTGGCTGGAA	GAAGCAGATA	ACATTGCTAT	TACTCCACTT	GGAGATGAGC	AGCAGCTAAA	6960
	AGAACAACCT	GAACAAGTCA	AGTTACTGGC	AGAAGAGTTG	CCCCTGCGCC	AGGGAATTCT	7020
	AAAACAATTA	AATGAAACAG	GAGGAGCAGT	ACTTGTAAGT	GCTCCCATAA	GGCCAGAAGA	7080
45	GCAAGATAAA	CTTGAAAAGA	AGCTCAAACA	GACAAATCTC	CAGTGGATAA	AGGTCTCCAG	7140
	AGCTTTACCT	GAGAAACAAG	GAGAGCTTGA	GGTTCACTTA	AAAGATTTTA	GGCAGCTTGA	7200
	AGAGCAGCTG	GATCACCTGC	TTCTGTGGCT	CTCTCCTATT	AGAAACCAGT	TGGAAATTTA	7260

SUBSTITUTE SHEET (RULE 26)

	TAACCAACCA	AGTCAGGCAG	GACCGTTTGA	CATAAAGGAG	ATTGAAGTAA	CAGTTCACGG	7320
	TAAACAAGCG	GATGTGGAAA	GGCTTTTGTG	GAAAGGGCAG	CATTTGTATA	AGGAAAAACC	7380
	AAGCACTCAG	CCAGTGAAGA	GGAAGTTAGA	AGATCTGAGG	TCTGAGTGGG	AGGCTGTAAA	7440
	CCATTTACTT	CGGGAGCTGA	GGACAAAGCA	GCCTGACCGT	GCCCCTGGAC	TGAGCACTAC	7500
5	TGGAGCCTCT	GCCAGTCAGA	CTGTTACTCT	AGTGACACAA	TCTGTGGTTA	CTAAGGAAAC	7560
	TGTCATCTCC	AAACTAGAAA	TGCCATCTTC	TTTGCTGTITG	GAGGTACCTG	CACTGGCAGA	7620
	CTTCAACCGA	GCTTGGACAG	AACTTACAGA	CTGGCTGTCT	CTGCTTGATC	GAGTTATAAA	7680
	ATCACAGAGA	GTGATGGTGG	GTGATCTGGA	AGACATCAAT	GAAATGATCA	TCAAACAGAA	7740
	GGCAACACTG	CAAGATTG	AACAGAGACG	CCCCCAATTG	GAAGAACTCA	TTACTGCTGC	7800
10	CCAGAATTG	AAAAACAAA	CCAGCAATCA	AGAAGCTAGA	ACAATCATT	CTGATCGAAT	7860
	TGAAAGAATT	CAGATTCAGT	GGGATGAGGT	TCAAGAACAG	CTGCAGAACA	GGAGACAACA	7920
	GTTGAATGAA	ATGTTAAAGG	ATTCAACACA	ATGGCTGGAA	GCTAAGGAAG	AAGCCGAACA	7980
	GGTCATAGGA	CAGGTCAGAG	GCAAGCTTGA	CTCATGGAAA	GAAGGTCCTC	ACACAGTAGA	8040
	TGCAATCCAA	AAGAAGATCA	CAGAAACCAA	GCAGTTGGCC	AAAGACCTCC	GTCAACGGCA	8100
15	GATAAGTGTA	GACGTGGCAA	ATGATTG	ACTGAAACTT	CTTCGGGACT	ATTCTGCTGA	8160
	TGATACCAGA	AAAGTACACA	TGATAACAGA	GAATATCAAT	ACTTCTTGGG	GAAACATTCA	8220
	TAAAAGAGTA	AGTGAGCAAG	AGGCTGCTTT	GGAAGAACT	CATAGATTAC	TGCAGCAGTT	8280
	CCCTCTGGAC	CTGGAGAAGT	TTCTTTCCTG	GATTACGGAA	GCAGAAACAA	CTGCCAATGT	8340
	CCTACAGGAC	GCTTCCCGTA	AGGAGAAGCT	CCTAGAAGAC	TCCAGGGGAG	TCAGAGAGCT	8400
20	GATGAAACCA	TGGCAAGATC	TCCAAGGAGA	AATTGAACT	CACACAGATA	TCTATCACAA	8460
	TCTTGATGAA	AATGGCCAAA	AAATCCTGAG	ATCCCTGGAA	GGTTCGGATG	AAGCACCCT	8520
	GTTACAAAGA	CGTTTGGATA	ACATGAATTT	CAAGTGGAGT	GAACITTCAGA	AAAAGTCTCT	8580
	CAACATTAGG	TCCCATTTGG	AAGCAAGTTC	TGACCAAGTGG	AAGCGTTTGC	ATCTTTCTCT	8640
	TCAGGAACCT	CTTGTTTGGC	TACAGCTGAA	AGATGATGAA	CTGAGCCGTC	AGGCACCCAT	8700
25	CGGTGGTGAT	TTCCAGCAG	TTCAGAAGCA	GAATGATATA	CATAGGGCCT	TCAAGAGGGA	8760
	ATTGAAAAC	AAAGAACCTG	TAATCATGAG	TACTCTGGAG	ACTGTGAGAA	TATTTCTGAC	8820
	AGAGCAGCCT	TTGGAAGGAC	TAGAGAACT	CTACCAGGAG	CCCAGAGAAC	TGCCTCCTGA	8880
	AGAAAGAGCT	CAGAATGTCA	CTCGGCTCCT	ACGAAAGCAG	GCTGAAGAGG	TCAACGCTGA	8940
	ATGGGACAAA	TTGAACCTGC	GCTCAGCTGA	TTGGCAGAGA	AAAATAGATG	AAGCTCTTGA	9000
30	AAGACTCCAG	GAACITTCAGG	AAGCTGCCGA	TGAACCTGGAC	CTCAAGTTGC	GCCAAGCTGA	9060
	GGTGATCAAG	GGATCCTGGC	AGCCAGTGGG	GGATCTCCTC	ATTGACTCTC	TGCAAGATCA	9120
	CCITGAAAAA	GTCAAGGCAC	TTCCGGGGAGA	AATTGCACCT	CTTAAAGAGA	ATGTCATCG	9180
	TGTCAATGAC	CTTGACATC	AGCTGACCAC	ACTGGGCATT	CAGCTCTCAC	CTTATAACCT	9240
	CAGCACTTTG	GAAGATCTGA	ATACCAGATG	GAGGCTTCTA	CAGGTGGCTG	TGGAGGACCG	9300
35	TGTCAGACAG	CTGCATGAAG	CCCACAGGGA	CTTTGGTCTC	GCATCCAGC	ACTTCTTTTC	9360
	CACITTCAGTT	CAGGGTCCCT	GGGAGAGAGC	CATCTCACCA	AACAAAGTGC	CCTACTATAT	9420
	CAACCACGAG	ACCCAAACCA	CTTGTTGGGA	CCACCCAAA	ATGACAGAGC	TCTACCAGTC	9480
	TTTAGCTGAC	CTGAATAATG	TCAGGTTCTC	CGCGTATAGG	ACTGCCATGA	AGCTCAGAAG	9540
	GCTCCAGAAG	GCCCTTTGCT	TGGATCTCTT	GAGCCTGTCA	GCTGCATGTG	ATGCCCTGGA	9600
40	CCAGCACAAC	CTCAAGCAAA	ATGACCAGCC	CATGGATATC	CTGCAGATAA	TTAACTGTTT	9660
	GACTACAATT	TATGATCGTC	TGGAGCAAGA	GCACAACAAT	CTGGTCAATG	TCCCTCTCTG	9720
	TGTGGATATG	TGTCTCAACT	GGCTTCTCAA	TGTTTATGAT	ACGGGACGAA	CAGGGAGGAT	9780
	CCGTGTCTCT	TCTTTTAAAA	CTGGCATCAT	TTCTCTGTGT	AAAGCACACT	TGGAAGACAA	9840
	GTACAGATAC	CTTTTCAAGC	AAGTGGCAAG	TTCAACTGGC	TTTTGTGACC	AGCGTAGGCT	9900
45	GGGTCTTCTT	CTGCATGATT	CTATTCAAAT	CCCAAGACAG	TTGGGTGAAG	TTGCTTCTCT	9960
	TGGGGGCAGT	AACATTGAGC	CGAGTGTGAG	GAGCTGCTTC	CAATTTGCCA	ATAATAAACC	10020
	TGAGATTGAA	GCTGCTCTCT	TCCTTGACTG	GATGCGCCTG	GAACCCAGT	CTATGGTGTG	10080

	GCTGCCCCGTC	TTGCACAGAG	TGGCTGCTGC	TGAAACTGCC	AAGCATCAAG	CCAAGTGTA	10140
	CATCTGTAAG	GAGTGTCCAA	TCATTGGATT	CAGGTACAGA	AGCCTAAAGC	ATTTTAATTA	10200
	TGACATCTGC	CAAAGTTGCT	TTTTTTCTGG	CCGAGTTGCA	AAGGGCCATA	AAATGCACTA	10260
	CCCCATGGTA	GAGTATTGCA	CTCCGACTAC	ATCCGGAGAA	GATGTTGCG	ACTTCGCCAA	10320
5	GGTACTAAAA	AACAAATTTT	GAACCAAAAG	GTATTTTGCG	AAGCATCCCC	GAATGGGCTA	10380
	CCTGCCAGTG	CAGACTGTGT	TAGAGGGGGA	CAACATGGAA	ACTCCCGTTA	CTCTGATCAA	10440
	CTTCTGGCCA	GTAGATTCTG	CGCCTGCCTC	GTCCCCCAG	CTTTCACACG	ATGATACTCA	10500
	TTACAGCATT	GAACATTATG	CTAGCAGGCT	AGCAGAAATG	GAAAACAGCA	ATGGATCTTA	10560
	TCTAAATGAT	AGCATCTCTC	CTAATGAGAG	CATAGATGAT	GAACATTTGT	TAATCCAGCA	10620
10	TTACTGCCAA	AGTTTGAACC	AGGACTCCCC	CCTGAGCCAG	CCTCGTAGTC	CTGCCAGAT	10680
	CTTGATTTCC	TTAGAGAGTG	AGGAAAGAGG	GGAGCTAGAG	AGAATCCTAG	CAGATCTTGA	10740
	GGAAGAAAAC	AGGAATCTGC	AAGCAGAATA	TGATCGCCTG	AAGCAGCAGC	ATGAGCATAA	10800
	AGGCCTGTCT	CCACTGCCAT	CTCCTCCTGA	GATGATGCCC	ACCTCTCCTC	AGAGTCCCAG	10860
	GGATGCTGAG	CTCATTGCTG	AGGCTAAGCT	ACTGCGCCAA	CACAAAGGAC	GCCTGGAAGC	10920
15	CAGGATGCAA	ATCCTGGAAG	ACCACAATAA	ACAGCTGGAG	TCTCAGTTAC	ATAGACTGAG	10980
	ACAGCTCCTG	GAGCAGCCCC	AGGCTGAAGC	TAAGGTGAAT	GGCACCACGG	TGTCCTCTCC	11040
	TTCCACCTCT	CTGCAGAGGT	CAGATAGCAG	TCAGCCTATG	CTGCTCCGAG	TGGTTGGCAG	11100
	TCAAACCTCA	GAATCTATGG	GTGAGGAAGA	TCTTCTGAGT	CCTCCCCAGG	ACACAAGCAC	11160
	AGGGTTAGAA	GAAGTGATGG	AGCAACTCAA	CAACTCCTTC	CCTAGTTCAA	GAGGAAGAAA	11220
20	TGCCCCCGGA	AAGCCAATGA	GAGAGGACAC	AATGTAGGAA	GCCTTTTCCA	CATGGCAGAT	11280
	GATTTGGGCA	GAGCGATGGA	GTCTTAGT	TCAGTCATGA	CAGATGAAGA	AGGAGCAGAA	11340
	TAAATGTTTT	ACAACCTCTG	ATTCCCGCAT	GGTTTTTATA	ATATTCTGTAC	AACAAAGAGG	11400
	ATTAGACAGT	AAGAGTTTAC	AAGAAATAAA	ATCTATATTT	TTGTGAAGGG	TAGTGGTACT	11460
	ATACTGTAGA	TTTCAGTAGT	TTCTAAGTCT	GTTATTGTTT	TGTTAACAAT	GGCAGGTTTT	11520
25	ACACGTCTAT	GCAATTGTAC	AAAAAAGTTA	AAAGAAAACA	TGTAAAATCT	TGATAGCTAA	11580
	ATAACTTGCC	ATTTCTTTAT	ATGGAACGCA	TTTTGGGTTG	TTTAAAAATT	TATAACAGTT	11640
	ATAAAGAGAG	ATTGTAAACT	AAAGTGTGCT	TTATAAAAAA	AGTTGTTTAT	AAAAACCCCT	11700
	AAACAAACAC	ACACGCACAC	ACACACACAC	ACACACACAC	ACACACACAC	GCACACATAC	11760
	ATGCACGAAC	CCACCACACA	CACACACACA	CACACACACA	CTGAGGCAGC	ACATTGTTTT	11820
30	GCATTACTTT	AGCGTGGTAT	TCATATGGAA	TTGATGACGT	TTTTTTTATTT	TCTTGCATAC	11880
	GAACCCACC	AAATGACTGC	TTGATATTGC	TCTTTTGAGA	ATTGTTGACT	GAGTGGGGCT	11940
	GGCTATGGGC	TTTCATTTTA	TACATCTATA	TGTCTACAAG	TATATAAATA	CTATAGGTAT	12000
	ATAGATAAAT	AGATATGAAG	TTACTTCTTC	AAATGTTCTT	GCCACTTCCT	AATGGAAATT	12060
	GCTTCTAGTC	ATCTGGGCTT	ATCTGCTTGG	GCAAGAGTGA	ATTTTCCCTG	GAGCCCAAAG	12120
35	CCAGGAGACT	ACCGCCACAC	TAAAATATTG	TCTAGGGCTC	CAGATGTTTC	TAGTTTTTAA	12180
	CTTTCCACTG	AGAGCTAGAG	GATTCATTTT	TTTCAAGGAA	CATGCGAATG	AATACACAGG	12240
	ACTTACTATC	ATAGTAATTT	GTTGGCTGAT	ATATTCAACT	TCCTACTGTT	GGGTTATATT	12300
	TAATGATGTT	TCTGCAATAG	AACATCAGAT	GACATTTTTA	ACTCCCAGAC	AGTAGGAGGA	12360
	AGATGGTAGG	AGCTAAAGGT	TGCGGCTCCT	CAGTCAATTT	ATATGAGGGG	AGCAACAAC	12420
40	CTGTAAAAGA	ATGGATGAAT	ATTTTACAAC	ATACATATAA	ACATCTCTAT	AATTACAAC	12480
	AAATTGTTCT	GCCCTCTTCA	TAAACTCAAC	CTGAAGTGGG	TGGTTTTGTT	GTTGTTGTTG	12540
	TTGTTGTTGT	TGATGATGAT	GATGAATTTT	AGATTTTAGA	TTTTTTGGGT	TTTTTTTTCT	12600
	TCATTGTGAT	GATTTTTTTT	TTTAATGCTG	CAAGACTTAG	GATTACTGTT	AAGAAAGTAA	12660
	CCCAATCACA	TTGTGACCC	GGTGAATATC	AGTCCAGAAG	CCCATGAACT	GCATTTGTCT	12720
45	CCTTTGCATT	GGTTTCCCTG	CAAGTAACTC	CACACAGGAT	TGTGGGTGAG	AAGGCACAGT	12780
	GGTTGGAAAG	TTTTGAGAGC	AAAAGCGTCT	CCAACTCTC	TGGTCTAGTT	GACGGGCTGA	12840
	AATGTCTAAA	CAAATGCAAG	TCATTGAACC	AGGAGAAAAA	GTGCAACAGA	AAGCTAAGGA	12900

SUBSTITUTE SHEET (RULE 26)

CTGCTAGGAA GAGCTTTACT CCTCTCATGC CAGTTTCCTC TTCTTAGCAT TTAAAGAGCA 12960
 TTCTCTCAAT AGAAATCACT GTCCTATCAT TTGCAAATC TGTACCTCT AACGTCAAGT 13020
 GTAATTAACT TCTAGCGAGT GGGTTTTGTC CATTATTAAT TGTAATTAAC ATCAAACACA 13080
 GCTTCTCATG CTATTTCTAC CTCACCTTGG TTTTGGGGTG TTTCTAGTAA TTGTGCACAC 13140
 5 CTAATTTTCAC AACTTCACCA CTGTCTGTT GTGTGGACAC CAGTTTCCTT TTTTCATTTA 13200
 TAATTTCCAA AAGAAAACCC AAAGCTCTAA GATAACAAAT TGAAATTTGG TTCTGGTCTT 13260
 GCTTTTCTCT CTCTCTCTCC TTTATGTGGC ACTGGGCATT TTCTTTATCC AAGGATTTGT 13320
 TTTACCAAG ATTTAAACA AGGGGTTTCT TTCTTACTAA GAAGTTTTAA GTTTCATTCT 13380
 AAAATCCAAG GTAGATAGAG TGCATAGTTT TGTMTAATC TTTTCGTTTT ATCTTTTAGA 13440
 10 TATTAGTTCT GGAGTGAATC TATCAAATA TTTGAATAAA AACTGAGAGC TTTATTGCTG 13500
 ATTTTAAGCA TAATTTGGAC ATCATTTTCT GTTCTTTATA ACCATCAAGT ATTAAGTGT 13560
 AAATCATAAT CAGTGTAAT GAAGCATAAT CATCACATGG CATGTATCAT CATGTCTCTC 13620
 AGGTACTGGA CTCTTACTTG AGTATCATAA TAGATTGTGT TTTAACACCA AACTGTGTAAC 13680
 ATTTACTAAT TATTTTTTTA AACTTCAGTT TTAGTGCATT TTCACAACAT ATCAGATTTT 13740
 15 ACCAAATATA TGCCTTACTA TTGTATTATA TTAGTGCTTT ACTGTGTATC TCAATAAAGC 13800
 ACGCAGTTAT GTTAC 13815

(2) INFORMATION FOR SEQ ID NO:18:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATACCGAGGC TGCACTGTAC A

21

30 (2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCAACCGAAC ATATCGCCAC GGCAGCCACC AACGATGCTG ATTGCCGTTT

50

40

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 57 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGTGGCTGCC GTGGCGATAT GTTCGGTTGC TAACITTACC CTTCATCACT AAAGGCC

57

5 (2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

15 AAACGTACAG CGCCATGTTT ACCAG

25

SUBSTITUTE SHEET (RULE 26)

CLAIMS

1. In a method for gene therapy, the improvement which comprises using a bacteriophage containing genetic material to introduce a therapeutic polynucleotide into a target cell of a mammalian recipient, wherein the mammalian recipient is diagnosed as having a condition that is treatable by administration to the recipient of the therapeutic polynucleotide or a product thereof, the method comprising the steps of:

(1) contacting the bacteriophage with the target cell under conditions to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and to allow the bacteriophage to enter the target cell; and

(2) allowing the target cell to live under conditions such that the therapeutic polynucleotide is transcribed therein,

wherein the bacteriophage is incapable of injecting said genetic material into the target cell of the mammalian recipient.

2. The method of claim 1, wherein the bacteriophage propagates in a prokaryotic cell.

3. The method of claim 1, wherein the bacteriophage includes exogenous genetic material that is transcribed and translated in the target cell.

4. The method of claim 1, wherein the bacteriophage is selected from the group consisting of a lambda phage, a P1 phage, a P22 phage, a T1 phage, a T2 phage, a T3 phage, a T4 phage, a T5 phage, a T6 phage, a T7 phage, a P2 phage, a P4 phage, an Mu phage, a PM2 phage, an N4 phage, an SPO1 phage, a PBS1 phage, and a PBS2 phage.

5. The method of claim 1, wherein the therapeutic polynucleotide is operably coupled to a promoter.

6. The method of claim 1, wherein contacting the bacteriophage with the target cell is performed in vitro.

7. The method of claim 1, wherein the bacteriophage contains exogenous genetic material that contains a cell-specific promoter that effects transcription and translation of the therapeutic polynucleotide in the target cell.

8. The method of claim 1, wherein the bacteriophage contains one or more modified tail proteins, wherein the modified tail proteins cannot facilitate injection of the genetic material into the target cell of the mammalian recipient.

9. A method for introducing an exogenous polynucleotide into a mammalian cell comprising:

(1) contacting a bacteriophage containing genetic material with the target cell under conditions to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and to allow the bacteriophage to enter the target cell; and

(2) allowing the target cell to live under conditions such that the therapeutic polynucleotide is transcribed therein,

wherein the bacteriophage is incapable of injecting the genetic material into the target cell.

10. The method of claim 9, wherein the bacteriophage includes exogenous genetic material that is transcribed and translated in the target cell.

11. The method of claim 9, wherein the bacteriophage is derived from a bacteriophage selected from the group consisting of a lambda phage, a P1 phage, a P22 phage, a T1 phage, a T2 phage, a T3 phage, a T4 phage, a T5 phage, a T6 phage, a T7 phage, a P2 phage, a P4 phage, an Mu phage, a PM2 phage, an N4 phage, an SPO1 phage, a PBS1 phage, and a PBS2 phage.

12. The method of claim 9, wherein the exogenous polynucleotide comprises a therapeutic polynucleotide.

13. The method of claim 9, wherein the bacteriophage contains one or more modified tail proteins, wherein the modified tail proteins cannot facilitate injection of the genetic material into the target cell of the mammalian recipient.

14. The method of claim 9, wherein contacting the bacteriophage with the target cell is performed in vitro.

15. The method of claim 9, wherein the bacteriophage has a genome that contains a cell-specific promoter that effects transcription and translation of the therapeutic polynucleotide in the target cell.

16. A bacteriophage comprising:

(a) a bacteriophage genome containing an exogenous polynucleotide that can be transcribed in a mammalian cell; and

(b) a ligand contained on the surface of the bacteriophage, wherein the ligand

selectively binds to a receptor expressed on the surface of a mammalian cell,

wherein the bacteriophage is incapable of injecting the bacteriophage genome into the mammalian cell.

17. The composition of claim 16, wherein the composition is contained in an implant that is suitable for implantation into a mammalian recipient

18. A bacteriophage comprising:

a bacteriophage containing a bacteriophage genome that can be transcribed in a mammalian cell, wherein the bacteriophage contains avidin on its surface and wherein the bacteriophage is incapable of injecting the bacteriophage genome into the mammalian cell.

19. The bacteriophage of claim 18, wherein the bacteriophage is contained in a kit with instructions for attaching a biotinylated ligand to the bacteriophage to form a ligand-labeled bacteriophage.

20. A kit comprising a container including:

(1) a first container containing a bacteriophage having a surface and a genome, wherein the bacteriophage is incapable of injecting the bacteriophage genome into a mammalian cell;

(2) a second container containing an agent for attaching a ligand to the surface of a bacteriophage, wherein the ligand binds to a receptor on the surface of the mammalian cell; and

(3) instructions for attaching the agent to the surface of the bacteriophage.

1/17

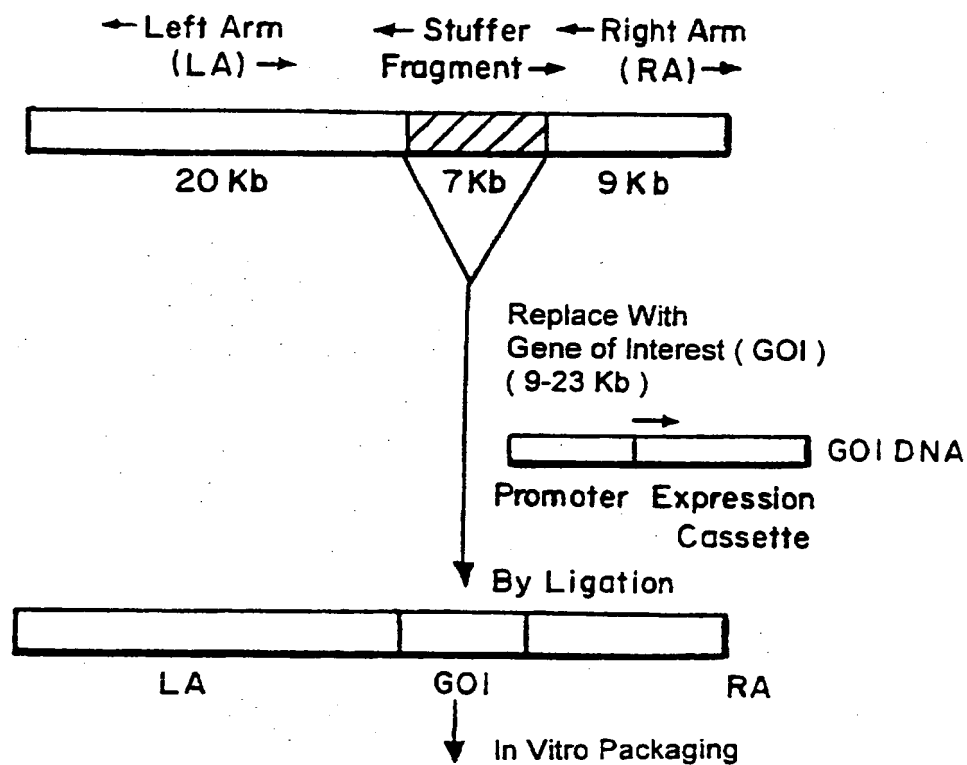


FIG. 1A

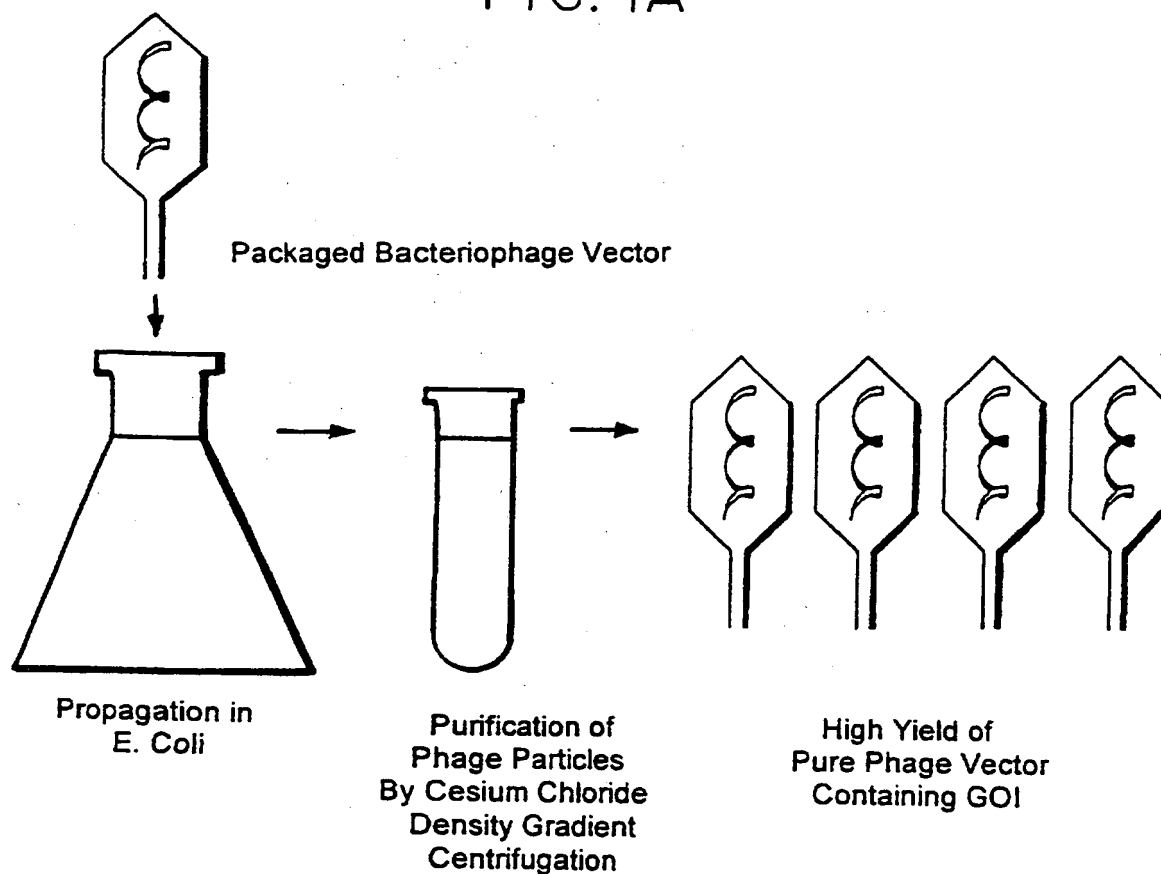
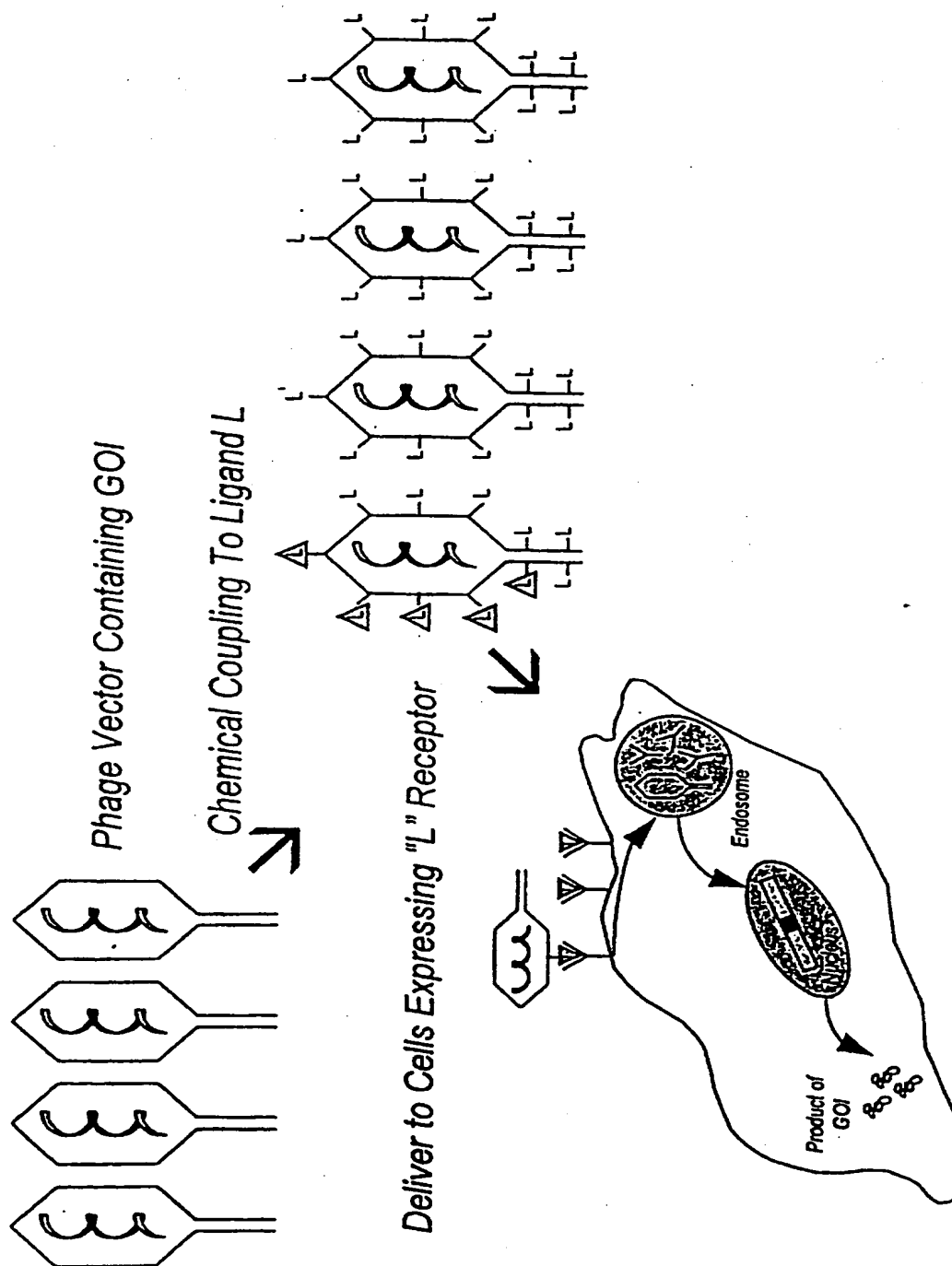


FIG. 1B

SUBSTITUTE SHEET (RULE 26)

2/17

Figure 1C

3/17

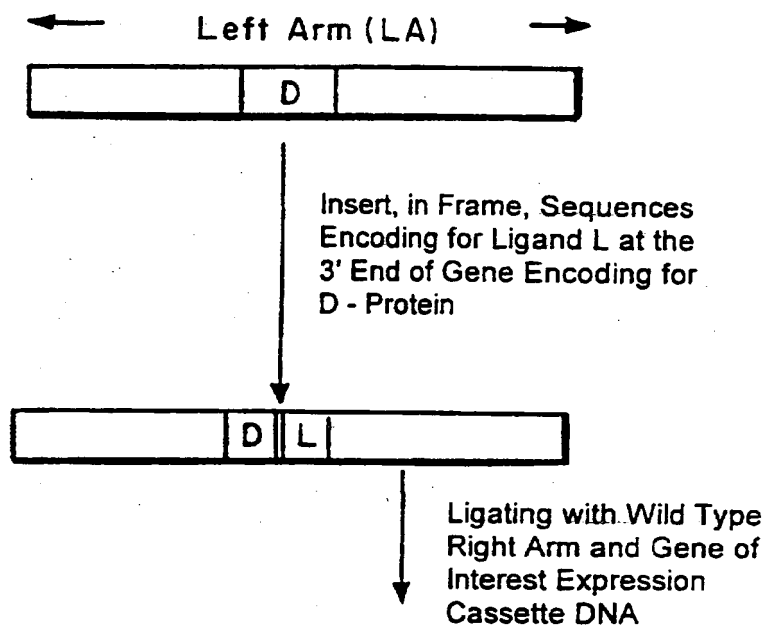


FIG. 2

4/17

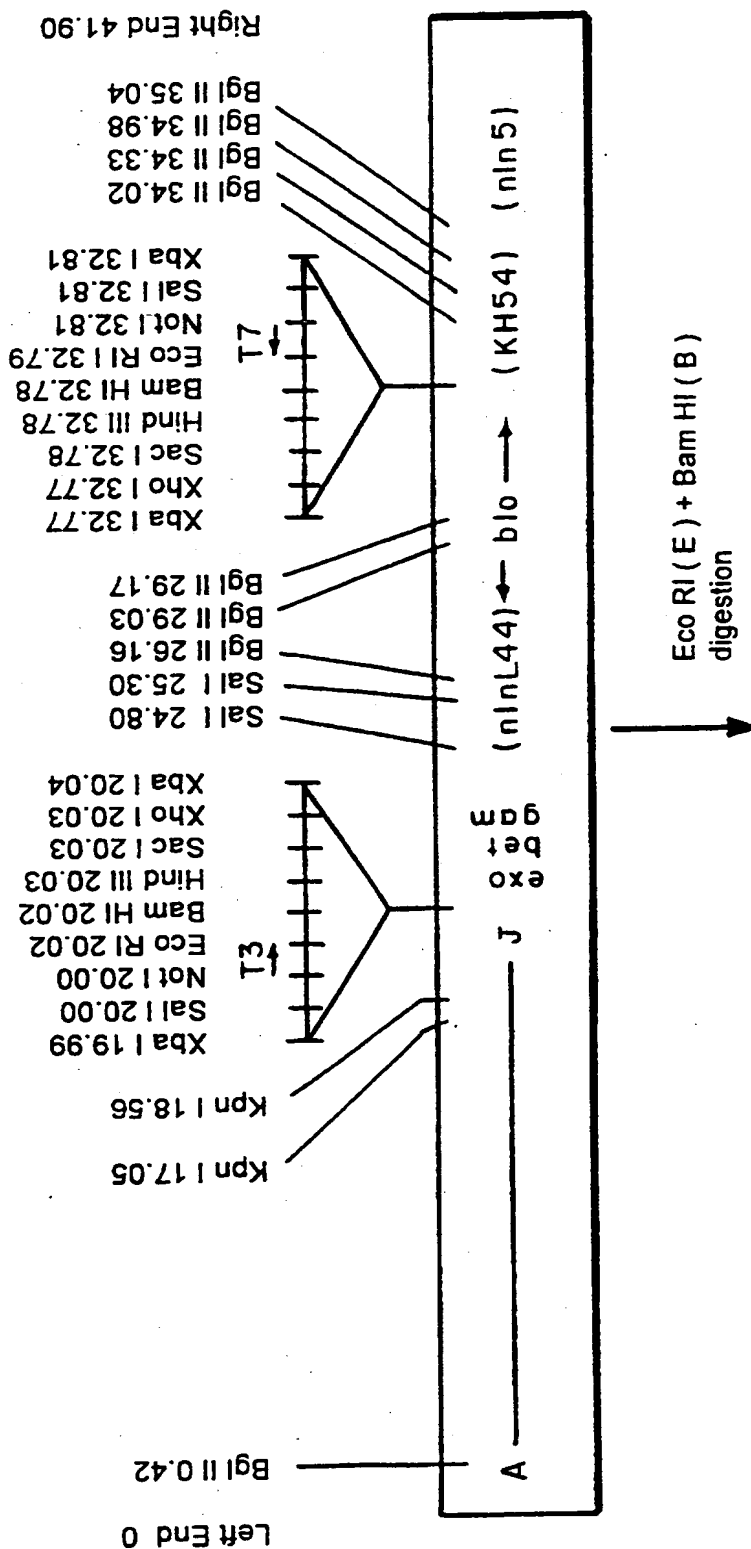


FIG. 3A

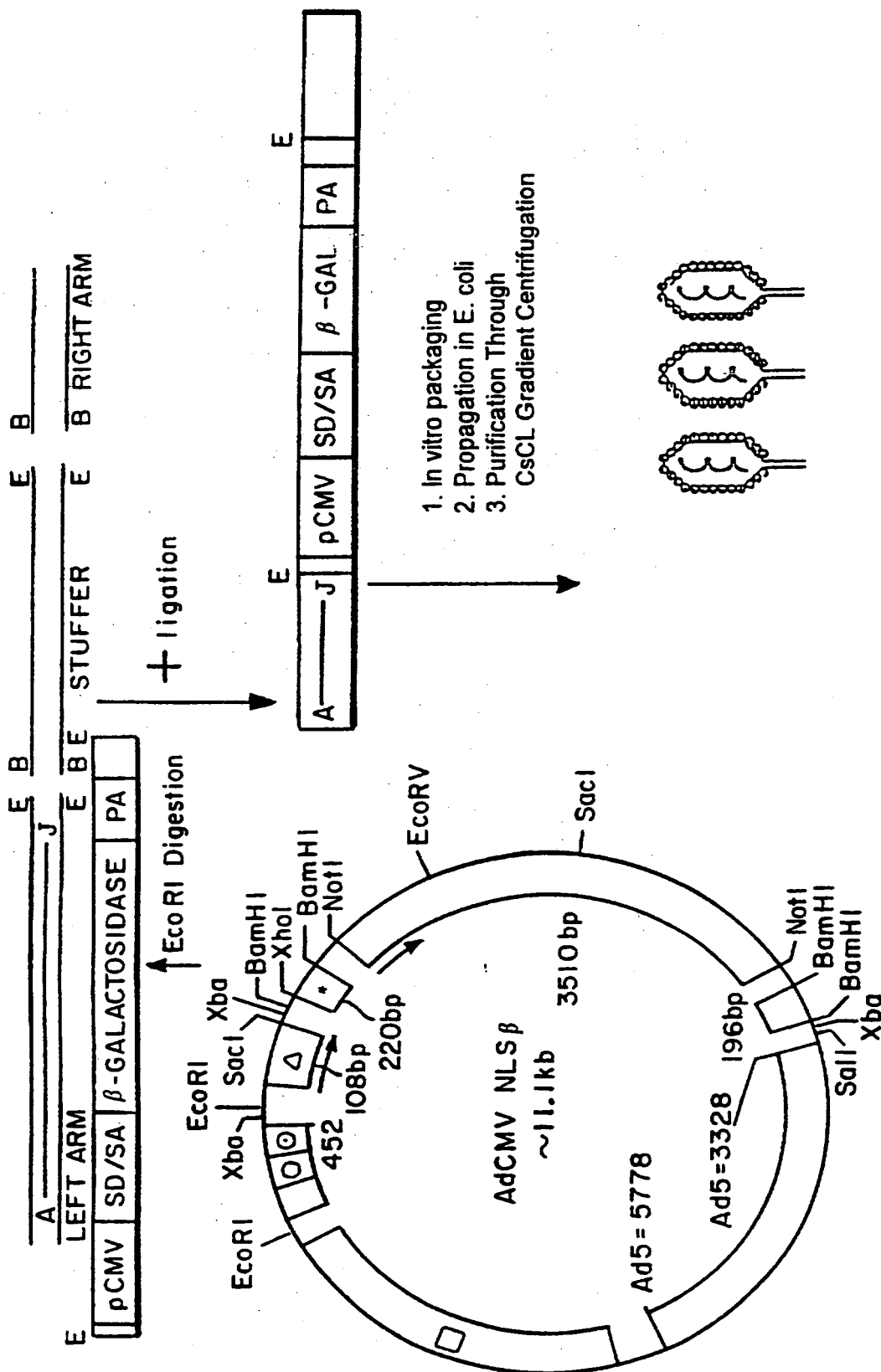


FIG. 3B

▲ pCMV = CMV enhancer / promoter
 * SD / SA = Splice donor / Splice acceptor SV 40
 # β - galactosidase (nuclear localized)
 ▴ Polyadenylation signal

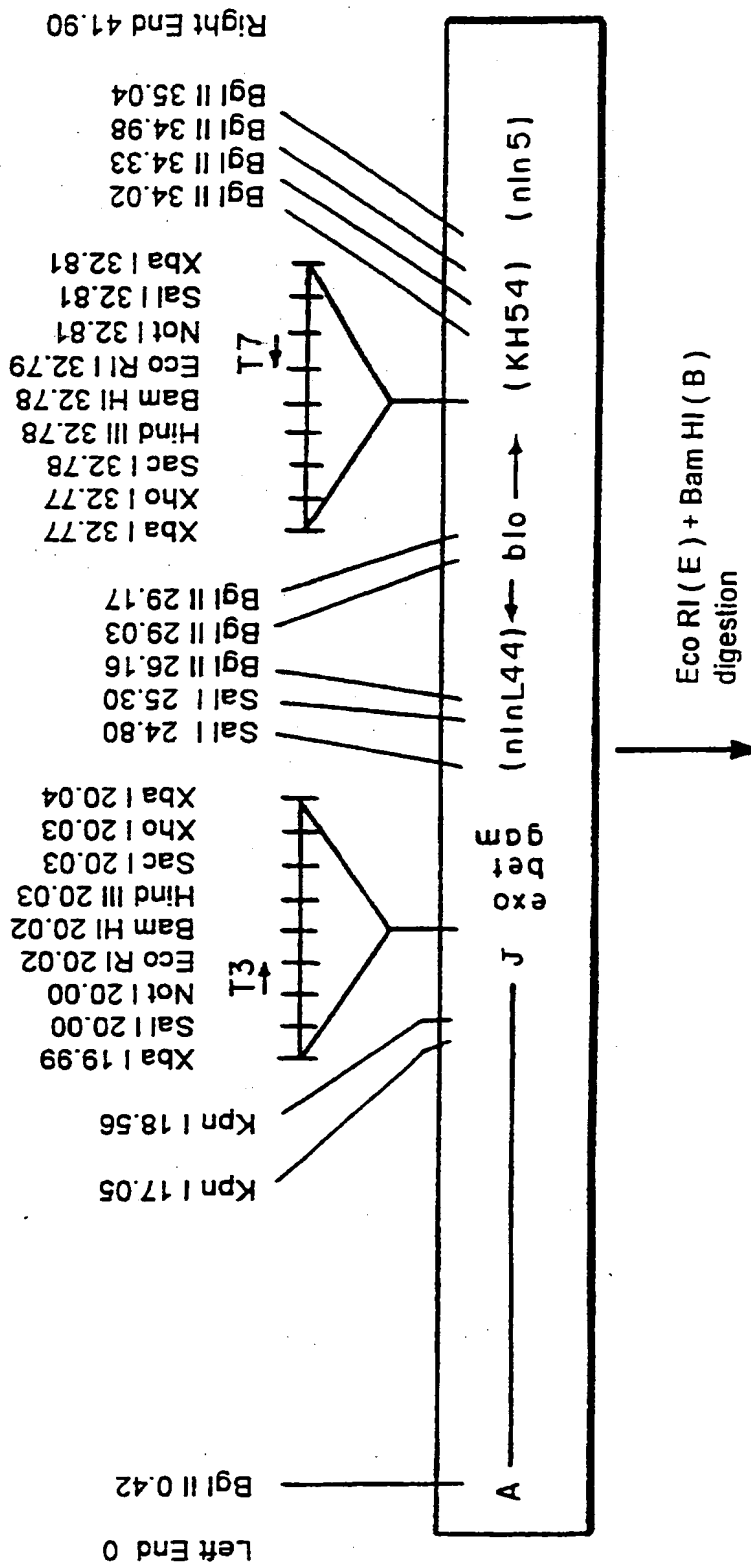


FIG. 4A

7/17

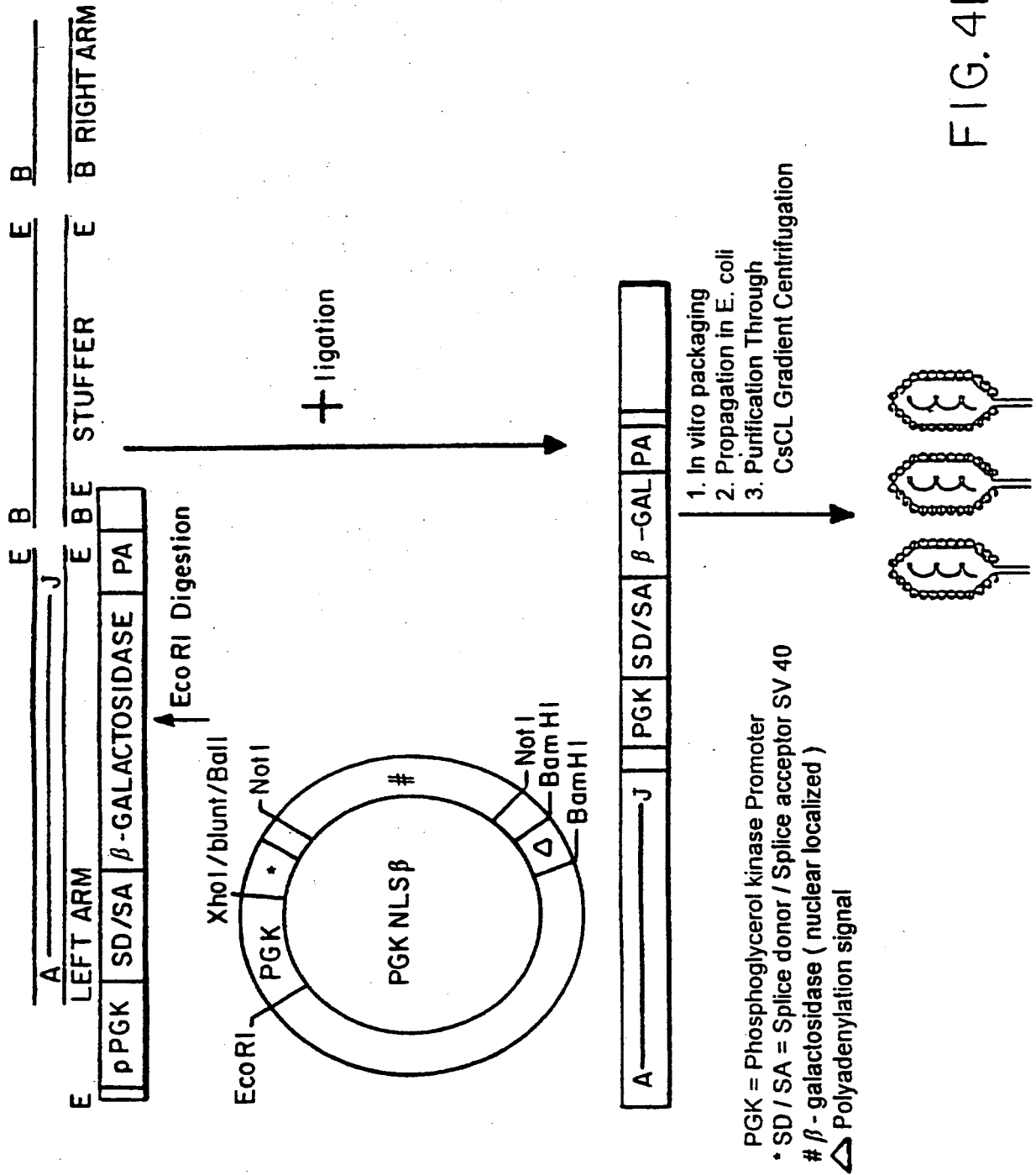


FIG. 4B

SUBSTITUTE SHEET (RULE 26)

8 / 17

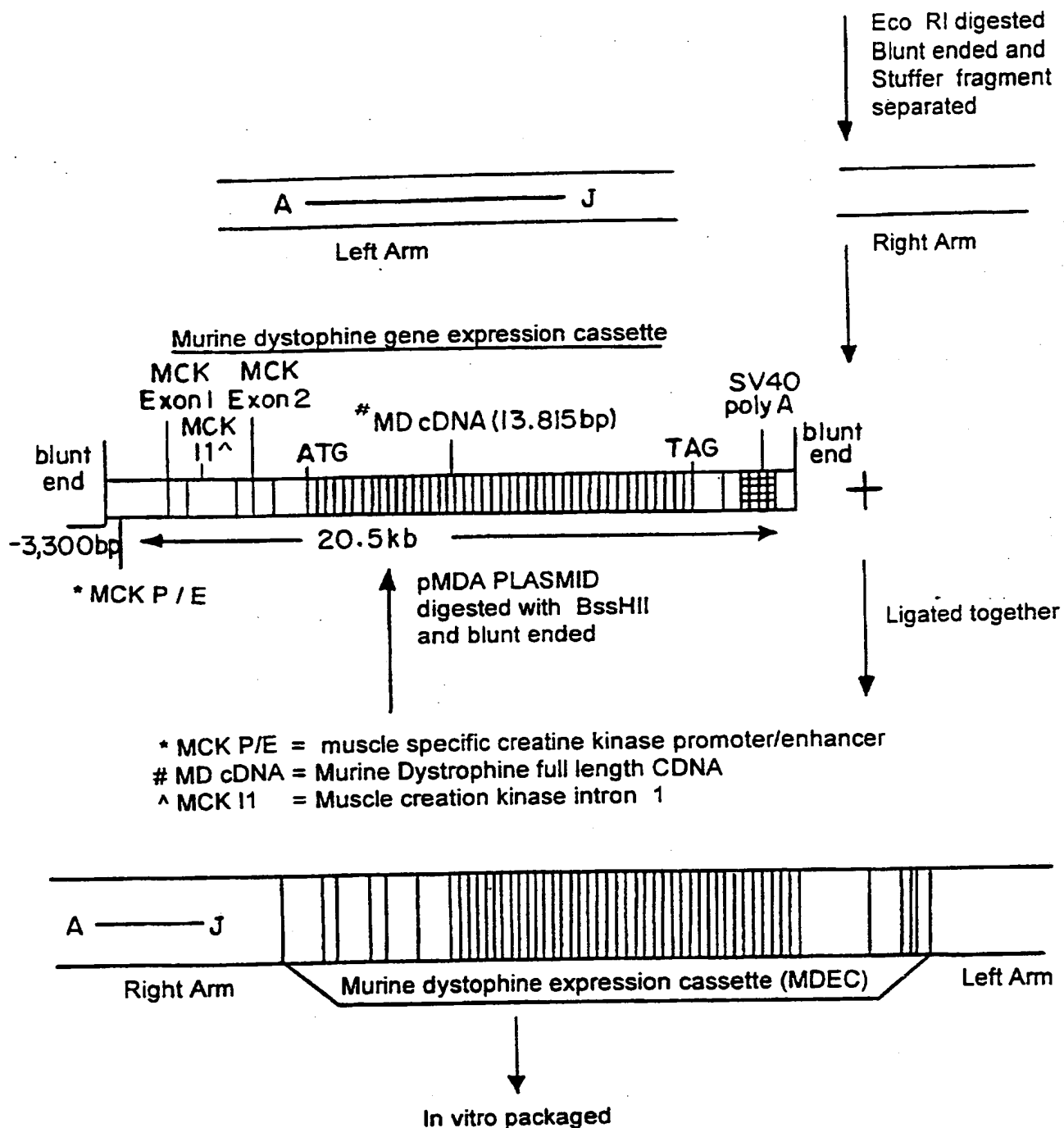
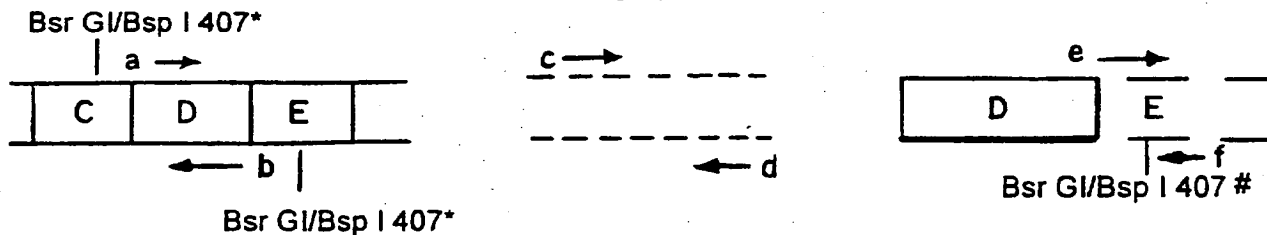


FIG.5

9/17



Wt λ genomic DNA template

Heregulin cDNA template

Wt λ genomic DNA template

* the location of this site is position 5220

the location of this site is position 6140

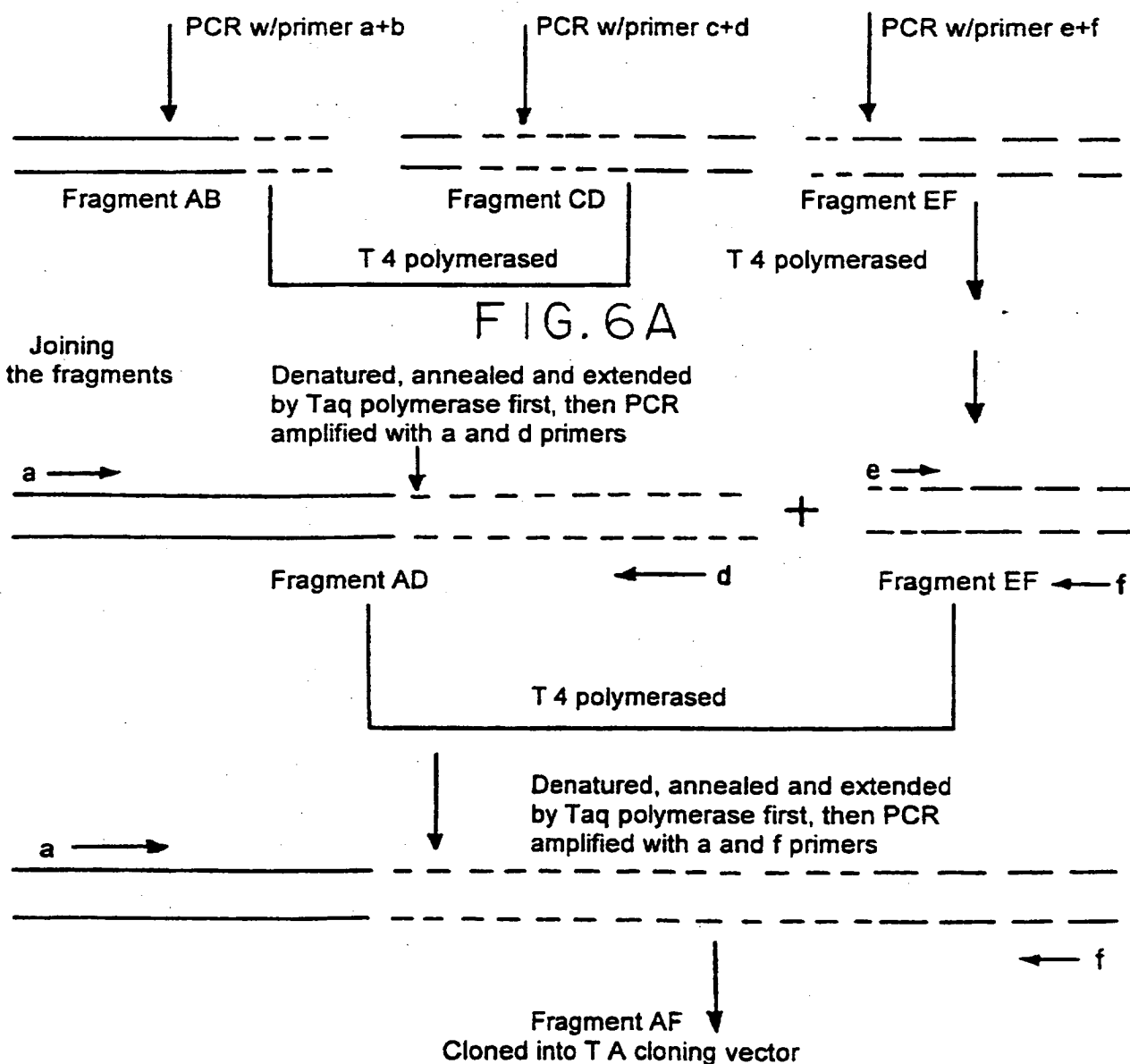
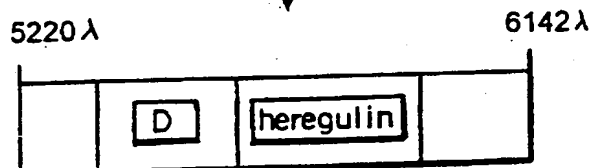


FIG. 6B
SUBSTITUTE SHEET (RULE 26)

10/17

T A plasmid containing fragment AF

digested with Bsr GI
and gel purified



ligated to Bsr GI digested
gel purified fragments of PGK/
β - gal/ λ DASH II DNA

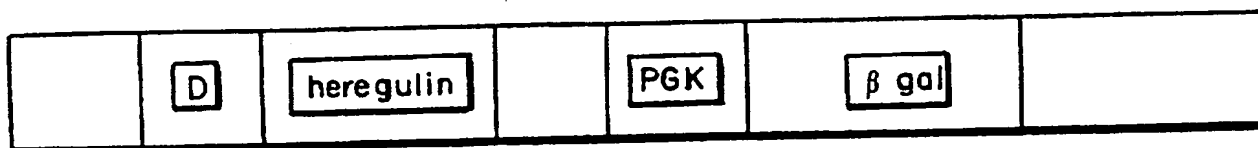
Fragment 1: *1-5220

Fragment 2: *5221-D- here - *6142

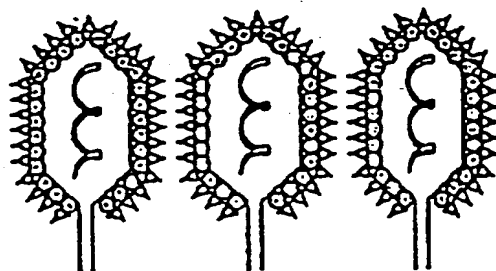
Fragment 3: *6143- 15855*

Fragment 4: *15856-PGK- β gal- λ *41900

* λ coordinates



Package with in vitro packaging extract



λ phage particle bearing a chimeric
D- ligand (L, heregulin) protein (DL)
on its capsid

FIG. 6C

Generation of targeted bacteriophage vectors by fusing a cyc
RGD ligand onto the bacteriophage λ virion head specific D - gene product

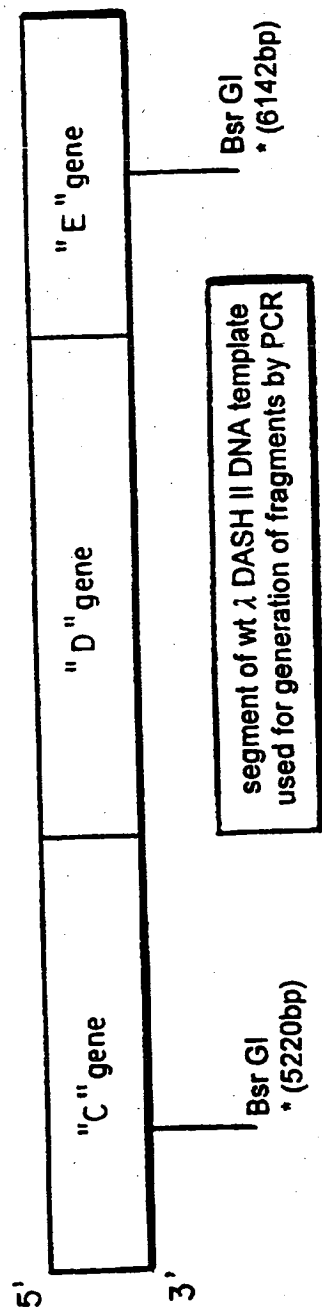


FIG. 7A

Using specially designed primers (as shown below), the cyc RGD coding sequences were fused onto the 3' end of the λ DASH II "D" gene as follows:

Generation of fragments for gene fusion

description of primers

"a" contains only wt sequence of the λ DASH I "C" GENE.

"b" has a 3' end that is complementary to 3' end of the wt "D" gene and a 5' end which contains the coding sequence to cyclic RGD.

"c" has a 5' end that is complementary to the 5' end of cyclic RGD and a 3' end which is complementary to the 5' end of the wt "E" gene.

"d" contains only wt sequence of the λ DASH II "E" gene

PCR generation and amplification of fragments

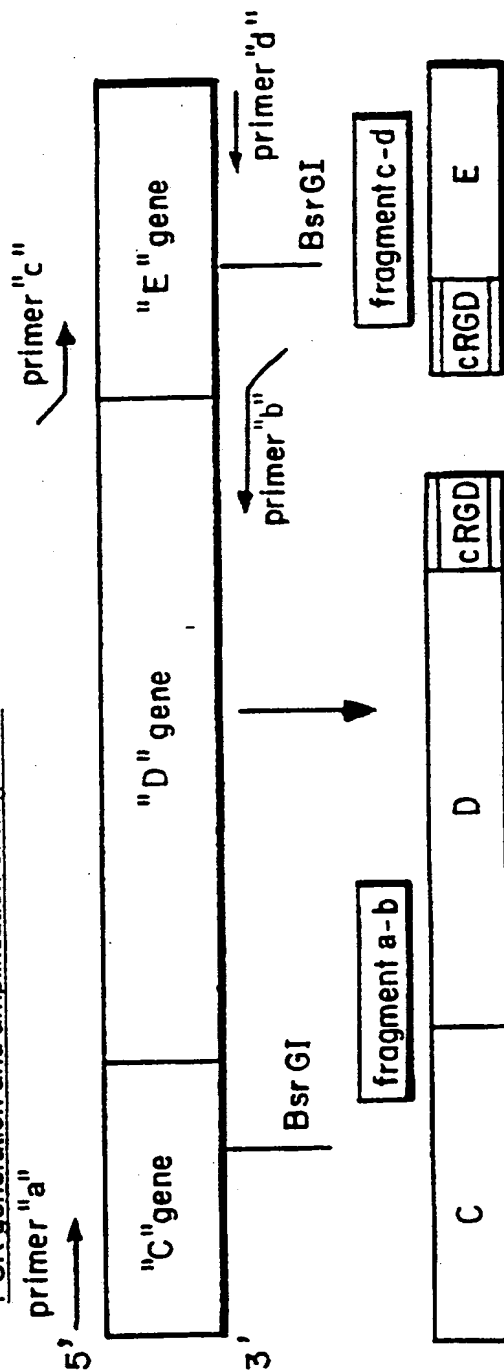


FIG. 7B

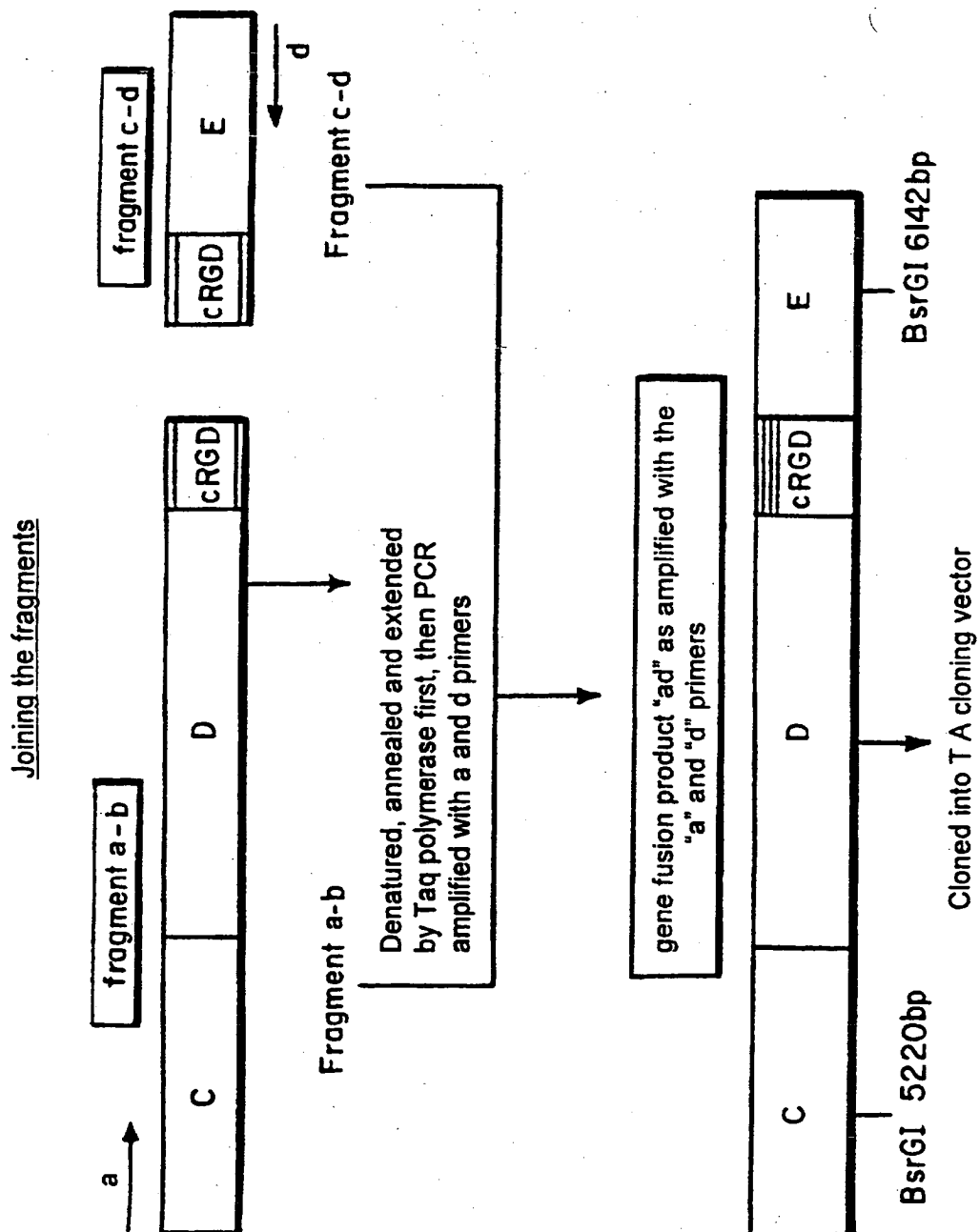


FIG. 7C

Generation of targeted λ phage vector expressing the cyc RGD-D chimeric capsid and containing the CMV β Gal reporter gene

preparation of λ DASH II / CMV β Gal vector

First, the λ DASH II / CMV β Gal bacteriophage vector was digested with Bsr GI and Eco RI* and cleaved into fragments. Fragment #2 (5220 - 6142bp) was then separated and removed by gel electrophoresis. (see below)

*co-digestion with Eco RI favors recombination of productive phage genome

Fragment 1: *1 - 5220

Fragment 2: *5221 - *6142

Fragment 3: *6143 - 15855*

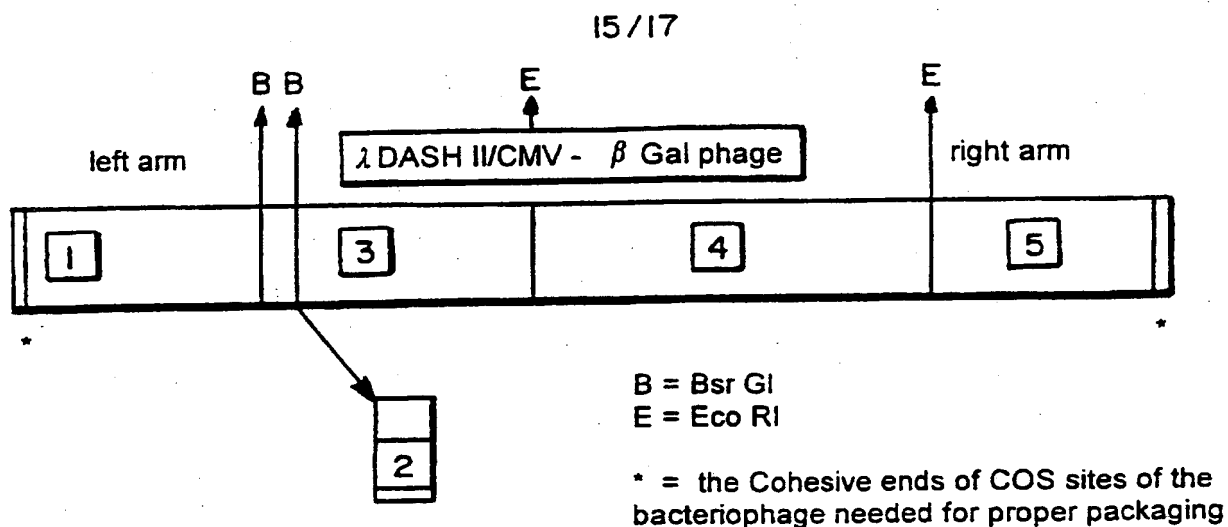
Fragment 4: *15856 - CMV - β gal - *41900

Fragment 5: *41900 - 48000

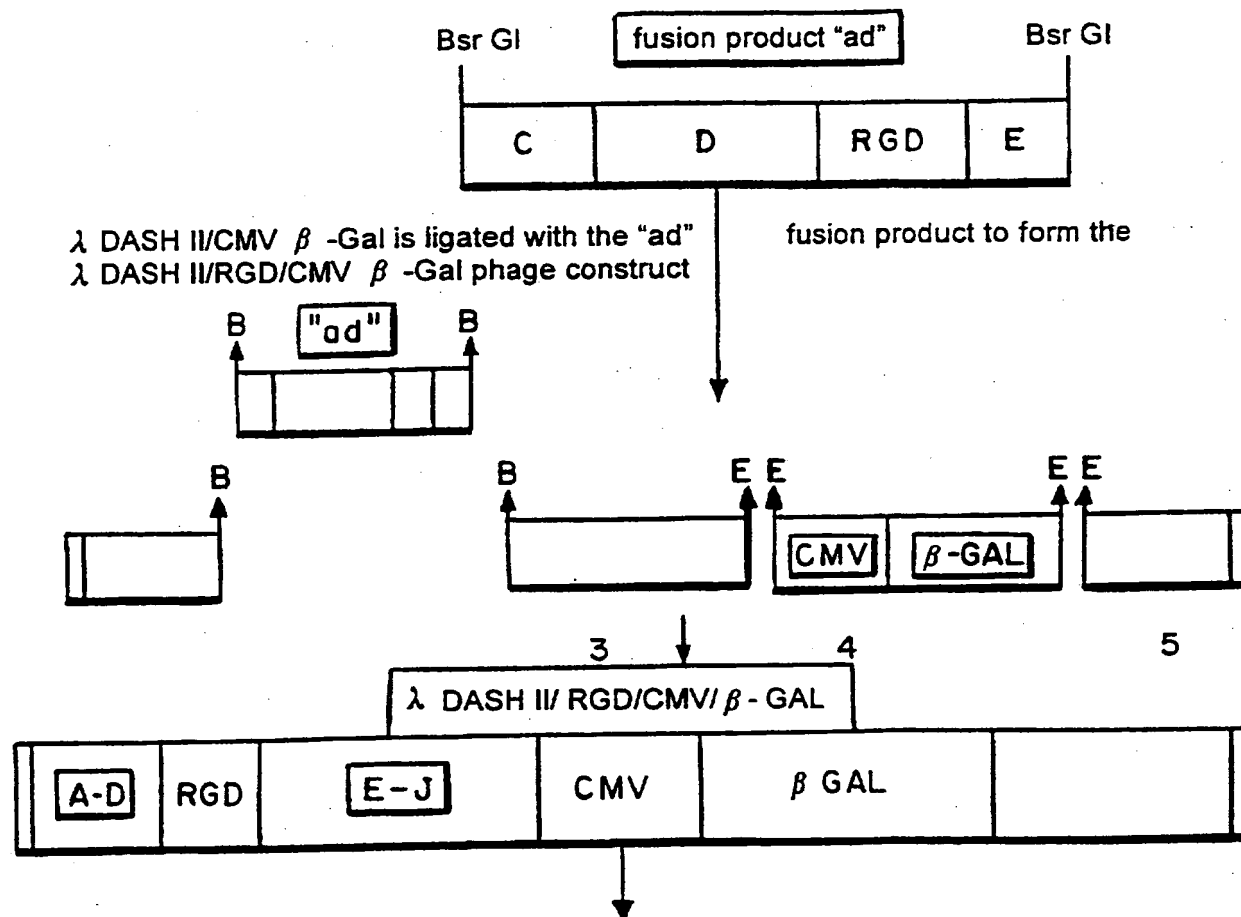
* λ coordinates



FIG. 7D-1



Next, the TA plasmid containing the "ad" gene fusion fragment is digested with Bsr GI



The ligated product was then packaged *in vitro* with *in vitro* packaging extract, the recombinant λ clones were characterized. Then λ DASHII/cyc RGD modified phage was then amplified and purified through CsCl banding

FIG. 7D-2
SUBSTITUTE SHEET (RULE 26)

16/17

This parental λ DASH II/cyc RGD/CMV β Gal modified bacteriophage was subsequently used as a vector for targeted gene transfer of other DNA constructs as described below

Generation of cyc RGD modified λ DASH II bacteriophage containing the murine dystrophin gene expression cassette

First, we ligated the COS sites of the cyc-RGD modified λ DASH II bacteriophage DNA together to protect them during the blunt ending process. Then the phage was digested with Eco RI and the CMV β Gal expression cassette was removed by gel electrophoresis.

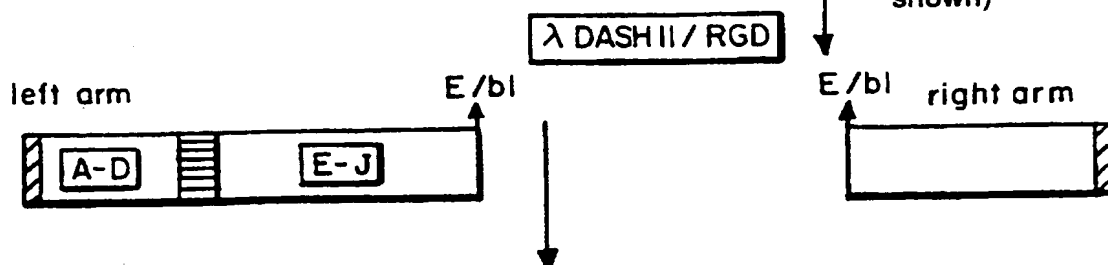
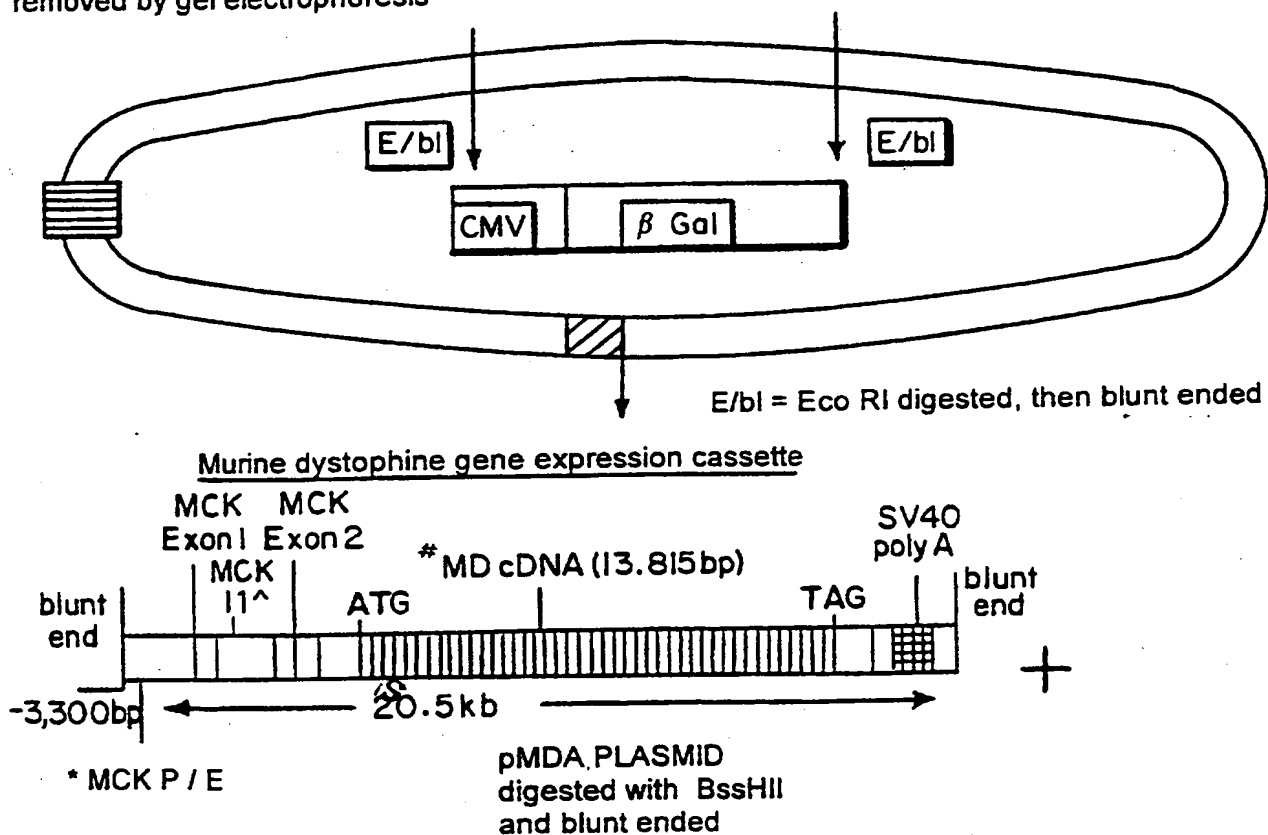


FIG. 7E
SUBSTITUTE SHEET (RULE 26)

17/17

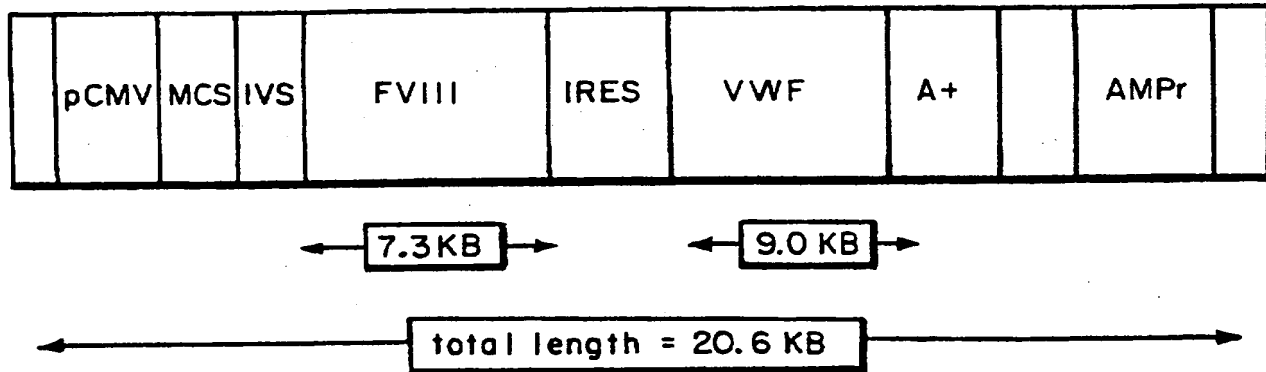


In vitro packaged, characterized, amplified and purified

Generation of cyc RGD modified λ DASH II bacteriophage containing the Factor VIII / IRES / Von Willebrand's Factor gene expression cassette

Construction of the plasmid containing the bicystronic expression cassette carrying the FVIII and the VWF genes

Gel purified, Eco RI digested, blunt ended, full length VWF cDNA was cloned into the Sma I site of the pIRES Δ neo (a derivative of the pIRESneo vector, in which the NEO gene was deleted by digestion with Kas I and Xba I and subsequent gel purification to remove the Kas I / Xba I fragment. The vector was then blunt ended and religated to generate the pIRES Δ neo vector (the parental pIRESneo was obtained from Clontech, CA.) The resulting plasmid, pIRES Δ neo / VWF, was then digested with Eco RI, blunt ended, and ligated to Xho / Sal digested, blunt ended and gel purified full length factor VIII cDNA fragment. The resulting plasmid (as shown below) constitutes the bicystronic expression cassette containing the FVIII / VWF genes.



Generation of the cyc RGD / λ DASH II / FVIII / IRES / VWF bacteriophage

Nru I digested pIRES Δ neo / FVIII / IRES / VWF plasmid DNA was used for ligation with the blunted left and right arms of the cyc RGD / λ DASH II prepared DNA. The ligated product was then *in vitro* packaged and clones were characterized, amplified and purified by CsCl banding.

FIG. 7F

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12928

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 35/76; C12N 7/00, 7/01, 15/09, 15/85, 15/86

US CL : 424/93.1, 93.6; 435/172.1, 172.3, 235.1, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.6; 435/172.1, 172.3, 235.1, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS
search terms: bacteriophage#, phage#, eukaryotic, eucaryotic, mammal###, human, mouse, transfect###, transduc####, gene therapy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/21007 (CHIRON VIAGENE, INC.) 11 July 1996 (11.07.96), page 4, line 30 to page 6, line 2, page 13, line 13 to page 22, line 14.	1-16
A	OKAYAMA et al. Bacteriophage lambda vector for transducing a cDNA clone library into mammalian cells. Molecular and Cellular Biology. May 1985, Vol. 5, No. 5, pages 1136-1142.	1-20
A	FLAMM et al. Expression of a cosmid containing the LCR, A-gamma, delta, and beta globin genes in mouse erythroleukemia cells. American Journal of Hematology. 1996, Vol. 53, pages 181-187.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

•	Special categories of cited documents:	"I"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
• "A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
• "E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
• "L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
• "O"	document referring to an oral disclosure, use, exhibition or other means		
• "P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

02 SEPTEMBER 1997

Date of mailing of the international search report
24 SEP 1997Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SCOTT D. PRIEBE

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12928

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HART et al. Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. Journal of Biological Chemistry. 29 April 1994, Vol. 269, No. 17, pages 12468-12474.	1-20

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: A61K 35/76, C12N 7/00, 7/01, 15/09, 15/85, 15/86	A1	(11) International Publication Number: WO 98/05344 (43) International Publication Date: 12 February 1998 (12.02.98)
(21) International Application Number: PCT/US97/12928 (22) International Filing Date: 3 July 1997 (03.07.97) (30) Priority Data: 08/693,865 5 August 1996 (05.08.96) US 08/814,859 11 March 1997 (11.03.97) US (71) Applicant (for all designated States except US): BRIGHAM AND WOMEN'S HOSPITAL, INC. [US/US]; 75 Francis Street, Boston, MA 02115 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SARKAR, Saumyendra, N. [IN/US]; 102 Queensbury Street, Boston, MA 02115 (US). KUPPER, Thomas, S. [US/US]; 8 Surrey Lane, Weston, MA 02193 (US). DUBIN, Daniel, B. [US/US]; Apartment D-618, 99 Pond Avenue, Brookline, MA 02146 (US). (74) Agent: PLUMER, Elizabeth, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: BACTERIOPHAGE-MEDIATED GENE THERAPY (57) Abstract An improved method for delivering an exogenous gene, e.g., a therapeutic polynucleotide, to a mammalian cell is provided. The method involves using a bacteriophage as a vector to deliver the exogenous gene to a pre-selected target cell. The bacteriophage contains exogenous genetic material that can be transcribed and, optionally, translated in a mammalian cell and includes on its surface a ligand that binds to a receptor on the target cell. The bacteriophage is incapable of injecting the exogenous genetic material into the mammalian cell. The bacteriophages are useful for gene therapy applications and for producing exogenous gene products <i>in vitro</i> .		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

BACTERIOPHAGE-MEDIATED GENE THERAPY

Government Support

15 This work was funded in part by the National Institutes of Health under the Grant Numbers AI 25062. The Government may retain certain rights in this invention.

Related Applications

This application is a continuation-in-part of U.S. Serial No. 08/814,859, filed March 11, 1989, pending, which is a continuation-in-part of U.S. Serial No. 08/693,865, filed August 5, 20 1996, pending, the entire contents of which are incorporated herein by reference.

Field Of The Invention

This invention relates to the use of a bacteriophage to express an exogenous gene in a mammalian cell. In particular, the invention relates to the use of bacteriophage vectors for human gene therapy and compositions related thereto.

Background Of The Invention

25 The application of gene therapy for the treatment of human disease has increased steadily since the first human gene therapy trial was conducted in 1989. To date, well over 100 (Science 269: 1050-5 (1995)) gene therapy protocols and clinical trials have been approved by the Recombinant DNA Advisory Committee for the treatment of inherited and acquired diseases. 30 Despite the reported advances in gene therapy technology and the increasing approvals of gene therapy protocols by the National Institutes of Health, the delivery and long-term expression of exogenous genes in specific tissues for the treatment of genetic disease remains a formidable challenge.

Vectors which are being studied in gene therapy trials include retroviruses, adenoviruses, 35 adeno-associated virus, plasmids and liposomes. Each has significant limitations. Although the adenovirus reportedly is a relatively efficient gene delivery vehicle for both dividing and non-dividing cells, its drawbacks include (1) limitation of inserted expression cassette to seven kilobases, (2) induction of inflammation in mammalian hosts, (3) toxicity to target cells when administered in high titers, (4) inability to target specific cell types, and (5) transient expression 40 in infected cells. Moreover, both injection of fully packaged adenovirus and subsequent expression of adenoviral antigenic proteins in infected cells provoke a host immune response that may significantly limit the bioavailability of repeated administration of the vector. Indeed, an inflammatory response to adenovirus during a cystic fibrosis gene therapy trial has been reported.

As an alternative to adenovirus, adenoassociated virus has been proposed as an

alternative to adenovirus, and does have certain advantages, including a preferential site of chromosomal integration. Although the adeno-associated virus has not been associated with inflammation, this virus' genome is only 4.7 kilobases in size and can accommodate inserts of up to 5 kb in size. Thus, its primary limitation is in the size of the "expression cassette" that it can deliver to the target cell. An "expression cassette" is a term of art that refers to an inserted exogenous DNA that optionally contains transcriptional regulatory sequences, translational regulatory sequences, coding sequences, or intervening sequences necessary for efficient expression of the included coding sequence. The cost of manufacturing adeno-associated virus is high compared to that of adenovirus. Furthermore, adeno-associated virus is difficult to grow at high titer and cannot be targeted to defined cellular targets.

The majority of gene therapy trials employ retroviral vectors for delivering an exogenous gene into mammalian cells and are susceptible to complement-mediated destruction. However, currently utilized retroviral vectors can transduce only dividing mammalian cells. In addition, the retroviruses can accommodate inserts up to only seven kilobases, thereby limiting the size of exogenous genetic material that can be delivered using this type of vector. In addition, the cost of manufacturing retroviral vectors has been estimated to be approximately \$100,000 per lot, reportedly due to the high costs associated with producing high titers of recombinant retroviral vectors in animal cells (D. Holzman, "Gene Therapy Depends on Finding the Right Vector", JNCI, Issue 6, vol. 87 (1995) NEWS pg 406). As late as 1995, only about half of the 76 retroviral clinical trials (Science 269: 1050-5 (1995)) approved for gene therapy had been initiated, in part, due to the high expense and difficulty in undertaking the manufacture of the retroviral vectors. The concern that retroviruses may replicate *in vivo* has inhibited clinical acceptance of retrovirus-mediated gene therapy and at least one incidence of replication of a retroviral vector occurring in clinical materials has been reported (D. Holzman, *ibid.*). There is also concern that random integration of retroviral vectors could disrupt or otherwise adversely affect host cell gene expression.

Non-viral vectors for gene therapy that are being studied in clinical trials include liposomes and naked DNA delivery systems. Low efficiency of transfection hampers both of these methods, neither of which permit targetable delivery to specific cell types. In addition, liposomes can be toxic to cells.

Despite the wide range of vectors currently available for human gene therapy, it is generally agreed that a clinically efficient and cost-effective vector for delivery of an exogenous

gene to specific mammalian cells or tissues has not been identified. The inadequacies of the above-identified vectors include: (1) induction of an immune response in the host; (2) possible deleterious recombination events; (3) limitations in the size of the expression cassette that can be inserted into the vector; (4) lack of specificity of the vector for delivering the expression cassette to a particular target cell; (5) inability to target a vector to both dividing and non-dividing cells; (6) high vector production cost, (7) low efficiency of transfection (non-viral vectors), (8) unacceptable toxicity, (9) low efficiency of expression *in vivo*, and (10) transience of expression of the exogenous genetic material. Accordingly it is incumbent upon scientists interested in realizing the unfulfilled promise of gene therapy to develop vectors that can overcome these shortcomings.

Summary Of The Invention

Applicants have discovered that certain well-characterized bacteriophages can be modified to contain exogenous genetic material that can be transcribed and, optionally, translated in a mammalian cell. Moreover, modification of these bacteriophages to include specific ligands and gene transcription regulatory elements permits control over the cell(s) to which the expression cassette is delivered and in which the delivered genetic material will be expressed. Furthermore, these bacteriophages can be modified to include nuclear localization and endosomal lysis signals to enhance the efficiency of transfection. Despite genetic modification, these bacteriophages preferably maintain the ability to propagate to high titer in a prokaryotic host, thus greatly facilitating production.

Thus, the invention provides compositions and methods for using these bacteriophages to introduce an exogenous polynucleotide (e.g., a therapeutic polynucleotide) into a pre-selected target cell via receptor-mediated delivery. According to one aspect of the invention, the bacteriophages are used to deliver a therapeutic polynucleotide into a mammalian cell for human gene therapy. In yet another aspect of the invention, the bacteriophages are used to deliver an exogenous polynucleotide into a mammalian cell for the production, *in vitro* or *in vivo*, of a polynucleotide transcription or translation product. According to yet other aspects of the invention, compositions containing the bacteriophages of the invention and methods for preparing and using the same to produce exogenous polynucleotide transcription and translation products *in vitro* also are provided. In certain preferred embodiments of the foregoing aspects of the invention, the bacteriophage contains exogenous genetic material that can be both transcribed and translated in a mammalian cell. In other preferred embodiments, the bacteriophage contains

exogenous genetic material that is transcribed, but not translated, in a mammalian cell (e.g., ribozyme and antisense constructs). Exemplary bacteriophages which can be modified to satisfy this criteria are provided in Table 1. (Tables 1-8 are presented at the end of the detailed description of the invention, immediately preceding the specific Examples section.)

Advantageously, these bacteriophages can be propagated in prokaryotic cell(s), thereby permitting the large scale production of the bacteriophages of the invention at a relatively low cost in comparison with other gene therapy vectors. Furthermore, since the bacteriophage structural genes are controlled by prokaryotic promoters, no transcription of native bacteriophage genes will occur in the mammalian host cell. Therefore, cells transfected with a modified bacteriophage will not express foreign proteins on their cell surface and, thus, will not induce cell-mediated immunity.

The preferred bacteriophages of the invention include the lambda and p1 phages. These preferred phages, after targeting modifications, have in common the ability to deliver to a mammalian cell(s) a therapeutic polynucleotide that is between about 1-100 kilobases. In particular, the preferred phages have in common the ability to deliver to a mammalian cell(s) a therapeutic polynucleotide of large size: between about 9-23 kilobases for lambda Dash II and between about 75-100 kilobases for p1. The preferred modified lambda phages of the invention can accommodate between about 9-50 kilobases. Thus, the invention is particularly useful for delivering to a target cell, an exogenous polynucleotide expression cassette (e.g., a therapeutic polynucleotide) containing a single gene or multi-gene complexes that are greater than 9 kilobases in length, something that cannot be accomplished by currently available viral vectors.

According to one aspect of the invention, an improved method for gene therapy is provided. The improvement lies in using a bacteriophage to deliver a therapeutic polynucleotide to a target cell in a mammalian recipient. In particular, the invention provides, for the first time, a viral vector that delivers to a target cell, an exogenous polynucleotide containing up to 9 to 23 kilobases for lambda Dash II, up to 50 kilobases for lambda-derived cassettes containing only COS sequences and no packaging protein coding sequences (minimal lambda cassette), and up to 75 to 100 kilobases for p1. The bacteriophages of the invention contain on their surfaces ligands to achieve delivery of the bacteriophages into a pre-selected target cell. The pre-selected target cell contains on its surface a receptor that selectively binds to the ligand, forming a ligand-receptor complex that is internalized by the cell. Although Applicants do not wish to limit the scope of the invention to a particular mechanism, it is believed that formation of the ligand-

receptor complex induces internalization of the complex via a receptor-mediated delivery mechanism such as that utilized by native eukaryotic viruses (e.g., adenovirus) and as that described in U.S. Patent No. 5,108,921, issued to Low, et al. and U.S. Patent No. 5,166,320, issued to Wu, et al., internalization of the bacteriophage into the target cell (i.e., transfection) can be performed *in vivo* or *ex vivo*. According to another aspect of the invention the bacteriophage vector further contains a nuclear localization signal (e.g., retrovirus) (Naldini et al. Science 1996; 272:263) as well as an endosomal lysis signal (e.g., adenovirus) (Wagner et al. Proc Natl Acad Sci 1992;89:6009) which, alone or in combination, enhance the expression of the delivered polynucleotide. Alternatively, chloroquine or other inhibitors of lysosomal/endosomal enzymatic catabolism can be co-administered with the vector. The use of nuclear localization signal(s), endosomal lysis signal(s), and/or endosomal enzyme inhibitors optimize expression of the delivered polynucleotide by minimizing the likelihood of DNA degradation that may occur as the vector traffics to the nucleus. In the particularly preferred embodiments, the delivered polynucleotide is designed and constructed in accordance with standard practice to integrate into the target cell chromosome. Alternatively, the vector may be maintained episomally in the cell. Accordingly, the novel bacteriophages disclosed herein are useful for *in vivo* and *ex vivo* gene therapy, as well as for producing in culture or in animals, gene products of the therapeutic polynucleotides (e.g., transcription products such as antisense RNA or catalytic RNA (e.g., ribozyme), and translation products such as peptides and proteins).

The improved method for gene therapy is useful for introducing a therapeutic polynucleotide (e.g., a polynucleotide for treating or diagnosing a medical condition) into a target cell of a mammalian recipient. The recipient is diagnosed as having a medical condition that is treatable by administration to the recipient of the therapeutic polynucleotide or a product thereof (e.g., a transcription product such as an antisense RNA or a translation product such a peptide or protein). The improved gene therapy method involves: (1) contacting the bacteriophage with the target cell under conditions (a) to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and (b) to allow the bacteriophage to enter the target cell; and (2) allowing the target cell to live under conditions such that the therapeutic polynucleotide is transcribed therein. Exemplary medical conditions and their respective therapeutic polynucleotides (or therapeutic polynucleotide products) that are useful for treating these medical conditions are provided in Table 2 (see, also, BioWorld Financial Watch, Monday Sept. 19, 1994, pp4-10, American Health Consultants, Inc.; Gene

Therapy A Primer for Physicians, Culver KW, Ed. 1996, Mary Ann Liebert, Inc. New York).

Therapeutic polynucleotides that are particularly suited for delivery using the bacteriophages of the invention are provided in Table 3. Exemplary target cells to which the bacteriophages can deliver these and other therapeutic polynucleotides are provided in Table 4. The preferred target cells express on their surfaces receptors that mediate cellular uptake of the bacteriophage vector via receptor-mediated endocytosis. More preferably, the target cells are non-phagocytic.

Screening methods for identifying target cells that internalize the bacteriophage vectors by way of receptor-mediated endocytosis are disclosed in the Examples. In general, these methods assay uptake by the target cell of the bacteriophage vector in the presence or absence of one or more known inhibitors of receptor-mediated endocytosis. The invention is directed to bacteriophage vectors that enter the cell via receptor-mediated endocytosis and not by the natural phage prokaryotic transduction pathway. Accordingly, in the preferred vectors, the bacteriophage tail proteins that are required for natural phage transduction are either, absent, non-functional in a prokaryotic host, or not capable of mediating injection of genetic material into a eukaryotic host cell. The screening methods disclosed in the Examples permit the selection of bacteriophage vectors and target cells which satisfy the above-noted criteria.

Advantageously, the preferred bacteriophage vectors are abortive to lytic growth in the natural bacterial flora of the mammalian host. Such modified bacteriophage vectors are also abortive to lytic growth in Su^0 (natural E. Coli host without the amber suppressor gene mutation *supE* or *supF*, both of which code for tRNAs) bacterial host strains in vitro. In the preferred embodiments, the bacteriophage vector genome is modified so as to contain an amber mutation inserted in-frame into a bacteriophage tail protein gene, e.g. J, M, or H gene, such that in an Su^0 bacterial host the tail protein gene is truncated and non-functional and, hence, abortive to lytic growth. Advantageously, the bacteriophage vectors with tail protein amber mutations propagate only in E. Coli strains containing either the amber suppressor gene mutation *supE* or *supF*.

Alternatively, the bacteriophage vectors have temperature-sensitive tail protein mutations abortive to lytic growth at mammalian host physiologic temperature. At temperatures other than (greater or less than) the mammalian host physiologic temperatures, the tail proteins are expressed and function normally and can mediate prokaryotic cell infections in vitro.

Accordingly, such modified bacteriophage vectors are packaged in vitro in cell free systems using purified protein packaging extracts and engineered cosmid vectors. In the preferred embodiments, the bacteriophage vectors have temperature-sensitive tail protein mutations such

that at temperatures other than (greater or less than) the mammalian host physiologic temperature, the tail proteins are expressed and function normally and can mediate prokaryotic cell infections; however, at mammalian physiologic temperature, the temperature sensitive mutation inactivates the wild type tail function.

5 In the preferred embodiments, the bacteriophage genome includes a promoter to control transcription and/or translation of the therapeutic polynucleotide in the target cell. Exemplary promoters are provided in Table 5. More preferably, the promoters are cell or tissue specific (i.e., they are functional only in particular types of cells or tissues), thereby providing an additional means for controlling expression (i.e., transcription and translation) of the therapeutic
10 polynucleotide in the target cell. Optionally, the bacteriophage genome includes additional regulatory sequences, e.g., enhancers, for further controlling expression of the therapeutic polynucleotide in the target cell. Exemplary enhancers also are provided in Table 5. In the preferred embodiments, the enhancers are target cell specific. Optionally, the bacteriophage genome can include eukaryotic origins of replication (e.g., from mammalian chromosomes),
15 telomeres and centromeres to permit autonomous replication of the bacteriophage genome within the target cell and segregation of the replicated genome into the target cell progeny.

The bacteriophage of the invention includes on its surface a ligand which selectively binds to a receptor on the target cell surface to form a ligand-receptor complex. The complex is internalized by the target cell, presumably by receptor-mediated endocytosis. Applicants do not
20 intend to limit the invention to a particular internalization mechanism, other than limiting the invention to exclude the natural phage transduction pathway that, as described in the literature, involves tail protein mediated injection of the bacteriophage genome into the target cell. The ligand can be attached to the surface of the bacteriophage using, for example, chemical modification methods, (e.g., galactosylation), genetic engineering methods (e.g., inserting a
25 sequence encoding the ligand into the bacteriophage genome, in frame, such that the ligand is expressed on the surface of the bacteriophage), specific adsorption (e.g., coating an antibody onto the surface of a bacteriophage) or a combination of genetic engineering and affinity binding methods (e.g., expressing avidin on the bacteriophage surface to form an "avidin-labeled
30 bacteriophage" and binding a biotinylated ligand thereto). Such avidin-labeled bacteriophages also are useful as intermediates in attaching virtually any ligand to the surface of a bacteriophage, provided that following biotinylation, the ligand retains its functional activity (i.e., the ability to selectively bind to its receptor to form a ligand-receptor complex). Potential ligands include

peptide or nucleotide polymers, macromolecular aggregates, such as lipoproteins or any chemical structure, either naturally occurring, synthesized, or generated by combinatorial chemistry techniques. Exemplary receptors and preferred ligands for targeting specific cell types are provided in Table 6. The preferred ligands include: low density lipoprotein (apoprotein B100), very low density lipoprotein (apoprotein E or a single chain variable immunoglobulin gene fragment that has high affinity for the VLDL receptor), HDL (apoA1), galactose, c kit ligand, transferrin, insulin, heregulin, and RGD or RGD-containing polypeptides (cyclic RGD).

The invention also embraces ligand-labeled bacteriophages in which the ligand is an antibody (or fragment thereof) that selectively binds to an antibody receptor (e.g., an Fc receptor) on the surface of a target cell. The antibody can be attached to the surface of the bacteriophage by, for example, selectively binding an antibody to a bacteriophage surface antigen. Thus, the invention is useful for targeting the delivery of an exogenous polynucleotide to virtually any Fc receptor bearing target cell *in vivo* or *ex vivo* via Fc receptor-mediated internalization. Finally, the avidin labeled phage can be bound to a biotinylated antibody. This antibody can bind to its cognate antigen on the cell surface, an event that is followed by patching, capping, and internalization. Optionally, the bacteriophage is modified, after *in vitro* propagation and packaging, to prevent or reduce the likelihood that the bacteriophage will enter bacteria that may be endogenous to the mammalian recipient or that may be a contaminant of target cells in culture. Such modifications can take the form of attaching the ligand to those portions of the bacteriophage (e.g., the "D" protein of the phage head) in a manner to inhibit the ability of the bacteriophage to attach to and/or penetrate its bacterial host. Alternatively, the ligand can be attached to the surface of a bacteriophage at a location that is not involved in phage attachment or penetration of the bacterial host. Other procedures, such as genetic engineering/chemical modification, can be used to interfere with the function of one or more bacteriophage encoded translation products that are essential for phage attachment and/or penetration into the bacterial host. (See, e.g., C. Merrill, et al., "Long-circulating bacteriophage as antibacterial agents", PNAS USA 93:3188-3192 (1996), which describes the importance played by the amino acid glutamic acid at position 158 of the capsid E protein in the insertion, *in vivo*, of bacteriophage lambda into *E. coli*).

According to yet another aspect of the invention, an avidin-labeled bacteriophage is provided. The bacteriophage has a genome in which the recombinant exogenous genetic material can be transcribed and translated in a mammalian cell. Since the native bacteriophage coding

sequences are regulated by prokaryotic promoters, in the ensuing discussion, the recombinant bacteriophage genome will refer only to the inserted genetic material whose expression is controlled by eukaryotic regulatory elements. Preferably, for bacteriophage vectors that are intended to produce a polypeptide, the recombinant bacteriophage genome can be both transcribed and translated in the mammalian cell. As mentioned above, the avidin-labeled bacteriophages are useful as intermediates in generating the ligand-labeled bacteriophages of the invention. Alternative high affinity binding pairs can be substituted for the avidin/biotin binding pair in accordance with the methods of the invention. For example, streptavidin can be substituted for avidin to form a streptavidin-labeled bacteriophage that can be allowed to bind to a biotinylated ligand to form a streptavidin-biotin complex. Alternatively, modified avidin or streptavidin may be employed.

According to yet another aspect of the invention, a method for introducing an exogenous polynucleotide into a target cell, preferably, a human cell, is provided. The method involves: (1) contacting the bacteriophage with the target cell under conditions: (a) to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and (b) to allow the bacteriophage to enter the target cell; and (2) allowing the target cell to live under conditions such that the exogenous polynucleotide is transcribed and, optionally, translated therein. As used herein, the phrase "exogenous polynucleotide" refers to a nucleic acid that is not normally present in the naturally-occurring (i.e., non-recombinant) bacteriophage. Thus, exogenous polynucleotides embrace the above-described therapeutic polynucleotides, as well as polynucleotides that are not intended for therapeutic applications (e.g., polynucleotides that encode a mammalian protein or protein complex for production of the protein or protein complex in cell culture, transcription regulatory elements, telomeres, centromeres, splice junctions, autonomous replicating sequences, recombination specific sequences). Exemplary bacteriophages and ligands that are useful in accordance with this method are described above in reference to the improved method for gene therapy. According to a particularly preferred embodiment, the method further involves the step of isolating an exogenous polynucleotide product (e.g., a transcription or translation product) from the target cell.

According to yet another aspect of the invention, a bacteriophage that is useful for practicing the above-described methods for delivering an exogenous polynucleotide (e.g., a therapeutic polynucleotide) to a target cell is provided. The bacteriophage, which can be propagated in prokaryotes, contains a recombinant genome that can be transcribed and,

optionally, translated in a mammalian cell. More preferably, the recombinant bacteriophage genome can be both transcribed and translated in the mammalian cell. The surface of the bacteriophage is modified to contain thereon a ligand that selectively binds to a receptor on the mammalian target cell.

5 The bacteriophages of the invention are useful in the preparation of a medicament for treating a medical condition that is treatable by administration to the mammalian recipient of the therapeutic polynucleotide or a product thereof. The bacteriophages can be placed in a pharmaceutically acceptable carrier to form a pharmaceutical composition which can be administered to the recipient in accordance with standard clinical practice known to one of
10 ordinary skill in the art. In a particular embodiment, the pharmaceutical composition is contained in an implant that is suitable for implantation in the mammalian recipient. Thus, the methods and compositions of the invention provide for an implantable bacteriophage gene therapy vector that is useful for delivering a therapeutic polynucleotide to the mammalian recipient over an extended period of time.

15 According to another aspect of the invention, a kit is provided which contains: (1) a first container containing an encapsidated bacteriophage having an appropriate surface marker and a genome (preferably, a genome having multiple cloning sites, such as the lambda DASH II genome) and instructions for inserting exogenous genetic material, which may contain coding sequence and upstream and downstream regulatory elements into the genome, preferable into the
20 multiple cloning site, (2) a second container containing an agent for attaching a ligand to the surface of the bacteriophage, wherein the ligand is designed to bind to a receptor on the surface of a mammalian cell; and (3) instructions for attaching the agent to the surface of the bacteriophage. More preferably, the kit further includes instructions for transducing a desired target mammalian cell. Alternatively, the desired ligand coding sequence is included in the
25 genome of the bacteriophage and the agent for attaching the ligand to the surface is unnecessary.

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments and to the accompanying drawings.

30 All references, patent publications and patents identified in this disclosure are incorporated in their entirety herein by reference.

Brief Description of the Drawings

Fig. 1, including 1A, 1B and 1C, is a schematic diagram describing the experimental strategy for generation of targeted bacteriophage vectors by chemical modification and use of the modified bacteriophage for gene delivery to cells of specific mammalian tissue origin.

Fig. 2 is a schematic diagram describing the experimental strategy for generation of targeted bacteriophage vectors by fusing the coding DNA sequences of a ligand "L" into the bacteriophage virion capsid specific "D" gene.

Fig. 3 is a schematic representation of a chimera of lambda DASH II /CMV promoter enhancer/beta-galactosidase gene sequences. Fig. 3A shows a restriction enzyme map and schematic representation of a restriction digest of the bacteriophage vector. Fig. 3B shows a schematic representation of the CMV promoter enhancer/beta-galactosidase gene sequences and ligation of this DNA to the digested bacteriophage to form the chimera, followed by (a) in vitro packaging, (b) propagation in *E. coli*, and (c) purification by CsCl gradient centrifugation to form a bacteriophage that is capable of expressing the beta-galactosidase (beta-gal) gene under the control of the CMV promoter in mammalian cells. The beta-gal gene contains a nuclear localization signal that directs localization of the translation product to the nucleus.

Fig. 4 is a schematic representation of a chimera of lambda DASH II/PGK promoter/-galactosidase gene sequences that are formed as described above in Fig. 3.

Fig. 5 is a schematic representation of a MCK/DMD/lambda bacteriophage chimeric DNA construct.

Fig. 6 is a schematic representation of a heregulin/lambda bacteriophage chimeric DNA construct in which a portion of the heregulin cDNA is fused, in frame, with the 3' end of the wild type capsid D-gene. Fig. 6A shows generation of the polynucleotide fragments; Fig. 6B shows the joining of the polynucleotide fragments; and Fig. 6C shows the generation of a modified bacteriophage expressing the heregulin-protein D chimeric capsid genes.

Fig. 7 is a schematic representation of A) the generation of targeted bacteriophage vectors by fusing a cyclic RGD ligand onto the bacteriophage lambda virion head specific D-gene product; B) generation of fragments for gene fusion; C) joining the fragments; D) generation of targeted lambda phage vector expressing the cyclic RGD-D chimeric capsid and containing the CMV beta-gal reporter gene; E) generation of cyc RGD modified lambda DASH II bacteriophage containing the murine dystrophin gene expression cassette; and F) generation of cyc RGD modified lambda DASH II bacteriophage containing the Factor VIII/IRES/Von

Willebrand's Factor gene expression cassette. In reference to fig. 7B, the primers are defined as follows: primer "a" (SEQ. ID NO. 18) contains only wt sequence of the lambda DASH II "C" gene; primer "b" (SEQ. ID NO. 19) has a 3' end that is complementary to the 3' end of the wt "D" gene and a 5' end which contains the coding sequence to cyclic RGD; primer "c" (SEQ. ID NO. 20) has a 5' end that is complementary to the 5' end of cyclic RGD and a 3' end which is complementary to the 5' end of the wt "E" gene; and primer "d" (SEQ. ID NO. 21) contains only wt sequence of the lambda DASH II "E" gene. In reference to fig. 7D, the preparation of the lambda DASH II/CMV beta-gal vector was by first digesting the vector with BsrGI and Eco R1 (*lambda coordinates) and cleaving into fragments. Fragment #2 (5220-6142bp) was then separated and removed by gel electrophoresis. Co-digestion with Eco R1 favors recombination of productive phage genome. (Fragment 1: *1-5220; Fragment 2: *5221-*6142; Fragment 3: *6143-*15855; Fragment 4: *15856-CMV-betagal-*41900; and Fragment 5: *41900-48000).

Detailed Description of the Invention

The instant disclosure provides bacteriophages for delivering an exogenous polynucleotide into a target cell, preferably a mammalian cell. A "bacteriophage", for the purposes of this invention, refers to a bacteriophage that: (1) contains exogenous genetic material that can be transcribed and, optionally, translated in a mammalian cell and (2) contains on its surface a ligand that selectively binds to a receptor on the surface of a target cell, such as a mammalian cell. As used herein, "exogenous genetic material" refers to a polynucleotide (e.g., nucleic acid or oligonucleotide), either natural or synthetic, that is not naturally found in a bacteriophage, or if it is naturally found in the bacteriophage, it is not transcribed or expressed at biologically significant levels by the bacteriophage. "Exogenous genetic material" includes a non-naturally occurring polynucleotide that can be transcribed into an anti-sense RNA, as well as all or part of a "heterologous gene" (i.e., a gene encoding a protein which is not expressed or is expressed at biologically insignificant levels in a naturally-occurring bacteriophage). Thus, for example, the instant invention embraces the introduction into a mammalian cell of an expression cassette including a recombinant gene containing an inducible promoter operably coupled to a coding sequence of a therapeutic polynucleotide. In the preferred embodiments, the exogenous genetic material of the bacteriophage can be both transcribed and translated in the mammalian target cell. Exemplary bacteriophages that satisfy at least the first of these criteria are provided in Table 1. Exemplary ligands that can be attached to the bacteriophage surface, e.g., covalently coupled to the surface, expressed, or specifically adsorbed or affinity bound thereto, are provided in Table 6. (Tables 1-8 are presented at the end of the detailed description of the invention, immediately preceding the specific Examples section.)

The bacteriophages are useful for delivering an exogenous polynucleotide into a mammalian target cell for *ex vivo* and *in vivo* gene therapy, as well as for producing exogenous polynucleotide products (e.g., transcription products such as antisense mRNA or catalytic RNAs and translation products) in culture or *in vivo*. The bacteriophages of the invention are particularly useful for delivering an exogenous polynucleotide containing between about one and one-hundred kilobases to a mammalian target cell, depending on the particular bacteriophage that is selected. For example, lambda and p1 can be used to deliver exogenous polynucleotides containing up to 9 to 23 kb and up to 75 to 100 kb, respectively. A minimal lambda cassette can deliver exogenous polynucleotides containing up to 50 kb. In contrast, conventional viral vectors for gene therapy viral vectors can accommodate, at best, a polynucleotide containing up to about

seven kilobases for delivery to a mammalian cell. Thus, the instant invention advantageously provides a method for delivering relatively large genes and/or multi-gene complexes to a mammalian cell for gene therapy purposes and for the *in vitro* or *in vivo* production of gene products. In general, the bacteriophages of the invention can be propagated in prokaryotic cells. Accordingly, the cost of producing the bacteriophages of the invention is relatively inexpensive compared to the cost of producing more conventional gene therapy vectors, such as retroviruses, adenovirus, or adeno-associated virus.

According to one aspect of the invention, an improved method for gene therapy is provided. The improved gene therapy method utilizes a bacteriophage as a vector to introduce a therapeutic polynucleotide into a target cell of a mammalian recipient. The improved gene therapy method involves two steps: (1) contacting the bacteriophage with the target cell under conditions (a) to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and (b) to allow the bacteriophage to enter the target cell; and (2) allowing the target cell to live under conditions such that the therapeutic polynucleotide is transcribed therein. The mammalian recipient is diagnosed as having a medical condition that is treatable by administration to the recipient of the therapeutic polynucleotide or a product thereof. In the preferred embodiments, the mammalian recipient is a human. Exemplary medical conditions and their respective therapeutic polynucleotides (or products thereof) that are useful for treating these conditions are provided in Table 2. In general, the medical conditions that are treatable in accordance with the methods of the invention include genetic diseases (i.e., diseases that are attributable to one or more gene defects) and acquired pathologies (i.e., pathological condition that are not attributable to an inborn genetic defect). The improved method for gene therapy also embraces prophylactic processes (i.e., delaying the onset of the foregoing medical conditions).

The bacteriophage genome contains a therapeutic polynucleotide that encodes a therapeutic polynucleotide product which is useful for treating (i.e., delaying the onset, inhibiting or reducing the symptoms of) the medical condition. As used herein, a "therapeutic polynucleotide" refers to a polynucleotide that mediates a therapeutic benefit in a recipient of the polynucleotide or product thereof. A therapeutic benefit may be an alteration of cell proliferation, a change of expression of a single or multiple genes or proteins, a cytotoxic effect against a pathogen, inhibition of viral replication, replacement of a defective gene and the like. Therapeutic polynucleotides may be administered in the form of a polynucleotide operably

joined to regulatory sequences, disposed in the bacteriophage vector for replication or regulated expression, or in separate non-operable pieces that can become operably joined in the target cell to yield an operable expression system. Therapeutic polynucleotides include genes encoding the transcription and translation products identified in Table 2. Therapeutic polynucleotides also embrace polynucleotides that encode diagnostic agents that can be detected *in situ* or *ex vivo* and that are useful in diagnosing a medical condition. Therapeutic polynucleotides that encode diagnostic agents include the genes encoding, for example, an enzyme that catalyzes a reaction, *in situ*, to yield a detectable product. Thus, as used herein, a "therapeutic polynucleotide product" refers to a molecule produced as a result of transcription or translation of the therapeutic polynucleotide. Therapeutic polynucleotide products include transcription products (e.g., antisense mRNA and catalytic RNA) and translation products (e.g., proteins or peptides) of the therapeutic polynucleotide.

Antisense oligonucleotides that have been approved for gene therapy protocols and/or clinical trials are provided in Table 2. As used herein, the phrases "antisense oligonucleotides" or "antisense" describe an oligoribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an RNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of the mRNA. The antisense molecules are designed so as to hybridize with the target gene or target gene product and thereby, interfere with transcription or translation of the target mammalian cell gene. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon the known sequence of a gene that is targeted for inhibition by antisense hybridization, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 7 and, more preferably, at least 15 consecutive bases which are complementary to the target. Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are

antisense to any region of the gene or RNA (e.g., mRNA) transcripts, in preferred embodiments the antisense oligonucleotides are complementary to 5' sites, such as translation initiation, transcription initiation or promoter sites, that are upstream of the gene that is targeted for inhibition by the antisense oligonucleotides. In addition, 3'-untranslated regions or telomerase binding sites may be targeted. Furthermore, 5' or 3' enhancers may be targeted. Targeting to mRNA splice sites has also been used in the art. In at least some embodiments, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., Cell Mol. Neurobiol. 14(5):439-457 (1994)) and at which proteins are not expected to bind. The bacteriophages of the invention, by virtue of their ability to accommodate therapeutic polynucleotides which are relatively large in size, are particularly useful for delivering to the target cell a polynucleotide that encodes multiple copies of the same or different mRNAs in tandem, thereby increasing the effective concentration of antisense mRNA in the target cell.

The selective binding of the antisense oligonucleotide to a mammalian target cell nucleic acid effectively decreases or eliminates the transcription or translation of the mammalian target cell nucleic acid molecule. Reduction in transcription or translation of the nucleic acid molecule is desirable in those medical conditions where transcription and translation of the mammalian target cell nucleic acid leads to an adverse medical condition. For example, the antisense oligonucleotides of the invention can be used to reduce the expression of oncogenes to treat cancers whose proliferation is mediated by expression of these oncogenes.

The bacteriophages of the invention are also useful for delivering therapeutic polynucleotides that encode specific antigen peptides to antigen presenting cells for processing and presentation at the cell surface to enhance the immune system response of the mammalian recipient to a specific peptide antigen. Exemplary peptide antigens that can be expressed to induce or otherwise enhance an immune response are shown in Table 7. In the preferred embodiments, the therapeutic polynucleotide encodes one or more peptide antigens that vaccinate the mammalian recipient against a tumor, a virus, a bacteria, or a parasite. Optionally, auxiliary therapeutic polynucleotides are inserted into the bacteriophage genome to enhance or otherwise improve the therapeutic efficacy of the therapeutic polynucleotide product in treating the condition. Exemplary auxiliary polynucleotides for delivery to the mammalian target cell include polynucleotides encoding tumor suppressor genes, polynucleotides encoding antisense mRNA or encoding catalytic RNA that inactivate oncogenes, and polynucleotides that render a

target tumor cell more susceptible to an administered drug (e.g., suicide genes encoding, for example, thymidine kinase). Auxiliary polynucleotides also include polynucleotides encoding cytokines that enhance a naturally occurring anti-tumor immunity. Exemplary cytokines which have this function include, e.g., IL-4, TNF, IL-2, and GM-CSF.

5 The therapeutic polynucleotide is inserted into the bacteriophage genome using conventional recombinant DNA techniques. See, e.g., *Methods in Enzymology*, vol. 152, ed. S. L. Berger, A.R. Kimmel (1987) Academic Press, New York, NY. In the preferred embodiments, the bacteriophage is a lambda phage and the therapeutic polynucleotide is inserted into well-defined restrictions sites in the lambda phage. (See, e.g., the Examples and figures.)
10 Optionally, recombination sequences (i.e., polynucleotides having a nucleic acid that allows homologous recombination) are provided at the 5' and 3' ends of the therapeutic polynucleotide to permit site-directed insertion of the therapeutic polynucleotide into a preselected location in the genomic DNA of the target cell via homologous recombination.

 In contrast to the viral vectors that presently are available for human gene therapy, the
15 bacteriophages of the invention can accommodate a therapeutic polynucleotide containing between about one and up to one-hundred kilobases, depending upon the particular bacteriophage selected. For example, lambda bacteriophages, e.g. lambda DASH II, and p1 phage can accommodate up to about 9 to 23 kb and up to about 75 to 100 kb, respectively. Minimal lambda cassette, such as described below, can accommodate up to about 50 kb.
20 Preferably, the therapeutic polynucleotide contains between about 10 and 90 kilobases, more preferably, the therapeutic polynucleotide contains between about 15 and 85 kilobases. Thus, the improved gene therapy method disclosed herein is particularly useful for gene therapy applications which require administration of a single therapeutic polynucleotide (or a product thereof) having a size within the foregoing kilobase range, as well as for delivering multiple
25 therapeutic polynucleotides which, together, have a size within this kilobase range. Exemplary therapeutic polynucleotides containing more than 7 kilobases include dystrophin, members of the globin gene complex, clotting factor VIII, von Willebrand's factor, collagen type VII, fibrillin, and any other gene(s)/gene complexes than are too large to deliver (efficiently) to mammalian cells using conventional viral vectors. Additional therapeutic polynucleotides that can be
30 delivered in accordance with the methods of the invention and that fall within the preferred kilobase size ranges can be identified by, for example, referring to the GenBank or other gene sequence data bases. See, also, Table 3 for a list of preferred therapeutic polynucleotides that can

be delivered using the bacteriophages disclosed herein. The delivery of a therapeutic polynucleotide containing more than 7 kilobases has not been possible using conventional gene therapy viral vectors. A summary of the insert size limitations for conventional gene therapy vectors compared to the bacteriophages disclosed herein is provided in Table 8.

5 The packaging and engineering of the lambda bacteriophage vector can be modified to permit the vector to accommodate up to approximately 50 kb of exogenous coding sequence. This approach involves engineering a recombinant cosmid vector DNA construct that contains an antibiotic resistance gene, e.g. ampicillin, a lambda origin of replication, and a DNA insert up to 50 kb in size flanked by COS (CoheSive ends of wild type bacteriophage lambda genome) sites. 10 This cosmid can be replicated to very high copy numbers in standard strains of E. Coli and then can be isolated using standard techniques for use in the packaging as described below.

The second component of this modified packaging system is a COS-negative lambda lysogen strain of bacteria in whose bacterial chromosome is integrated the structural proteins and enzymes requisite for packaging of an infective lambda virus. Using standard recombinant 15 techniques (Molecular Cloning, 2nd Edition, Sambrook et al., Cold Spring Harbor Laboratory, 1989), the lysogen strain is engineered to contain modifications of certain packaging proteins such that the final modified lambda phage vector is able to effectively target the intended cell type(s), sub-cellular compartments, or sub-cellular organelles. For example, E. Coli can be infected with a modified bacteriophage that contains a fusion D gene-RGD construct. Using 20 standard methods lysogen that contain the D gene-RGD fusion construct can be selected. Combining protein extracts from a D gene minus lysogen with a D gene-RGD fusion lysogen will provide the full complement of necessary packaging proteins. Using this combination of lysogen extracts, bacteriophage genomes or minimal lambda cassettes can be encapsidated in vitro with a D gene-RGD fusion protein that can target the recombinant virus to cells expressing 25 RGD's cognate receptor. Preferably, the D-gene is modified to include in frame a ligand, e.g. cyclic RGD, so that the vector is internalized by the targeted cell type(s). This lysogen strain is incapable of producing virus because the COS sites are absent. The lysogen strains are grown to large quantities using standard bacterial culture techniques. Then, the lambda packaging proteins, including any modified forms of these proteins, can be obtained by standard methods, 30 such as freeze thawing and sonication of lysogen.

The packaging of the high capacity bacteriophage lambda vector is accomplished by mixing the purified engineered cosmid DNA with the isolated protein extract from the above

lysogen strain at approximately room temperature. This mixing results in the packaging of replication deficient modified bacteriophage lambda virus particles that contain both surface proteins as determined by the modified lysogen strain from which the packaging proteins are derived and a genome of an insert of up to about 50 kb flanked by COS sites yet lacking other bacteriophage coding sequences.

In the preferred embodiments, the bacteriophage genome further includes a regulatory sequence, e.g., a promoter region (also referred to as a "promoter"), that is operably coupled to the therapeutic polynucleotide. The regulatory sequence controls the expression of the therapeutic polynucleotide in the target cell. As used herein, a therapeutic polynucleotide (also referred to as "coding sequence") that encodes a therapeutic polynucleotide product, and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the transcription or the expression of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequence results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 3' or 5' non-transcribed and/or non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, CAAT sequence, and the like. Especially, such 5' non-transcribing regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream 5' or downstream 3' transcriptional regulatory sequences as desired. The bacteriophages of the invention optionally include 5' leader signal, or membrane integration sequences 5' or 3'.

Exemplary promoters that are useful for protecting the instant invention are provided in Table 5, which shows both constitutive promoters and regulatable promoters (e.g., cell lineage

specific promoters, inducible promoters). Exemplary constitutive promoters also are included in Table 5. In the preferred embodiments, the constitutive promoter is selected from the group consisting of a promoter of the phosphoglycerokinase gene, a long terminal repeat (LTR) of retroviruses, e.g., Rous sarcoma virus, Moloney murine leukemia virus. Exemplary tissue or cell specific transcriptional regulatory sequences are derived from the genes encoding the following proteins: tyrosinase, lipoprotein lipase, albumin, muscle creatine kinase, keratin (K14/K10), globin gene cluster, immunoglobulin heavy chain gene cluster, and involucrin. Several liver-specific promoters, such as the albumin promoter/enhancer, also have been described (see, e.g., PCT application number PCT/US95/11456, having international publication number WO96/09074, entitled "Use of a Non-mammalian DNA Virus to Express an Exogenous Gene in a Mammalian Cell," hereinafter W0 96/09074, and the references cited therein). In particular, the alpha-feto protein promoter, can be used to effect expression of a therapeutic polynucleotide(s) in liver tumor cells (but not normal liver cells) for treating liver cancer. Exemplary inducible promoters are identified in Table 5 and are described in the following references: Science 268:1786 (1995); TIBS 18:471 (1993); PNAS 91:3180 (1994); PNAS 90:1657 (1993); PNAS 88:698 (1991); Nature Biotechnol. 14:486 (1996); and PNAS 93:5185 (1996). The preferred inducible promoter system is the tetracycline inducible system. An exemplary repressible promoter, the tetracycline repressible system, is identified in Table 5 and is described in PNAS 89:5547 (1992).

A tetracycline inducible promoter system includes, for example, two tandem constructs: an appropriate promoter operably linked to a trans-activating polypeptide coding sequence (rtTA) (a mutated Tet R linked to a VP16) and poly A signal in tandem with a tetracycline responsive element (tetO and a eukaryotic minimal promoter) operably coupled to a coding sequence of interest. Containing a poly A site, these two tandem constructs can be, optionally, joined into a single construct separated by an internal ribosomal entry site (IRES). The tetO driven coding sequence is 5' to the mammalian promoter driven coding sequence. Addition of tetracycline, doxycycline, or derivatives thereof, activates the transactivating polypeptide to bind tetO and, in turn, to drive transcription of a polycistronic message including first the coding sequence of interest and the transactivator. The IRES allows initiation of translation of the trans-activating polypeptide or the coding sequence of interest, independently, from a single polycistronic message. In the absence of tetracycline, the transactivator is not activated and the tetO driven transcription of the coding sequence of interest is substantially reduced to negligible

levels.

Preferably, the bacteriophage genome further includes an enhancer region ("enhancer"). Exemplary enhancers that are useful for practicing the instant invention are provided in Table 5. The preferred enhancers are selected from the group consisting of the following: a locus control region (beta-globin enhancer), an immunoglobulin gene enhancer, a cytomegalovirus (CMV) enhancer, a muscle creatine kinase enhancer, and an SV40 enhancer. Optionally, the bacteriophage genome can be engineered to contain an origin of replication to effect autonomous replication and facilitate persistence of the therapeutic polynucleotide in the mammalian cell. Origins of replication derived from mammalian target cells have been identified (see, e.g., Burhans, et al., 1994, Science 263: 639-640).

The bacteriophages optionally contain one or more sequences that are suitable for use in the identification of cells that have or have not been transfected. "Transfection", as used herein, refers to the introduction of the bacteriophage genome into the target cell. Markers to identify cells that have been transfected include, for example, genes encoding proteins that increase or decrease resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes having activities that are detectable by standard assays known in the art and genes which detectably (e.g. visibly) effect the phenotype of the transfected target cells, hosts, or plaques. Exemplary genes that are suitable as markers include the lacZ genes, chloramphenicol acetyltransferase genes, alkaline phosphatase genes, luciferase genes, and green fluorescent protein genes.

The bacteriophages of the invention contain on their surface a ligand that selectively binds to a receptor expressed on the surface of the target cell. The bacteriophage ligand binds to the target cell receptor to form a ligand-receptor complex that is internalized by the target cell. In this manner, the bacteriophage can be targeted for delivery to a pre-selected cell or tissue type, i.e., by selecting a ligand/receptor pair wherein the receptor is selectively expressed on a specific population of cells. Exemplary pairs of ligands/receptors which are useful in accordance with the methods of the invention are provided in Table 6.

The preferred ligand/receptor pairs for use in accordance with the methods of the invention include the following: insulin/insulin receptor, heregulin/heregulin receptor, keratinocyte growth factor/keratinocyte growth factor receptor, hepatic growth factor/hepatic growth factor receptor, RGD peptides/integrin alpha-5:beta-1, interleukin-2/interleukin-2 receptor, galactose/asialoglycoprotein, low density lipoprotein (LDL) or apoB100/LDL receptor,

very low density lipoprotein (VLDL), apoE/VLDL receptor, or HDL or apoA1/HDL receptor. In addition to these ligand/receptor pairs, antibodies can be attached to the bacteriophage via interaction of a bacteriophage coat protein specific antibody with its cognate antigen or via interaction of a recombinant bacteriophage coat protein that contains avidin and a biotinylated antibody. Once the antibody is tightly bound to the bacteriophage as described above, the antibody can direct the bacteriophage either to cells that express the Fc receptor in the case of a coat protein specific IgG antibody or to cells that express the cognate antigen of the attached biotinylated antibody. Selective ligand-receptor interaction also is useful for mediating internalization of the bacteriophage into subcellular locations e.g., the nucleus, mitochondria, and other membranes-bound organelles or cytoplasmic molecular aggregates of protein and/or nucleic acid.

In addition to the well-known ligand/receptor pairs for delivering a ligand-labeled component to a particular cell type, novel ligands can be identified using phage display procedures such as those described in (S. Hart, et al., J. Biol. Chem. 269(17):12468 (1994)). While such filamentous phages could, of course, never be used to deliver genetic material to a cell (because they are single stranded), this methodology is potentially very useful in the discovery of novel receptor ligand interactions. In general, phage display libraries using, e.g., M13 or fd phage are prepared using conventional procedures such as those described in the foregoing reference. The libraries display inserts containing from 4 to 80 amino acid residues. The inserts optionally represent a completely degenerate or a biased array of peptides. Ligands that bind selectively to a particularly type of target cell (e.g., mammalian cell) are obtained by selecting those phages which express on their surface a ligand that binds to the target cell of interest. These phages then are subjected to several cycles of reselection to identify the peptide ligand-expressing phages that have the most useful binding characteristics. Typically, phages that exhibit the best binding characteristics (e.g., highest affinity) are further characterized by nucleic acid analysis to identify the particular amino acid sequences of the peptides expressed on the phage surface and the optimum length of the expressed peptide to achieve optimum binding to the target mammalian cell. Alternatively, such peptide ligands can be selected from combinatorial libraries of peptides containing one or more amino acids. Such libraries can further be synthesized which contain non-peptide synthetic moieties which are less subject to enzymatic degradation compared to their naturally-occurring counterparts. These novel ligands can be attached to the bacteriophage surface to deliver the phage to the particular target cell of

interest. Alternatively, the ligands may be selected from polynucleotide libraries, as polynucleotides can also demonstrate specific affinity for polypeptide cell surface receptors. Exemplary screening methods for selecting bacteriophage vectors that transduce mammalian target cells via receptor-mediated endocytosis and target cells that internalize the bacteriophage vectors by this mechanism are described in the Examples.

At least four general procedures are available for attaching a ligand to the surface of a bacteriophage. These include (1) chemical modification of the bacteriophage surface (e.g., galactosylation, cross linking reactions); (2) modification of the bacteriophage genome to express a ligand on the bacteriophage surface (e.g., a fusion protein formed between the ligand and a functional viral packaging protein); (3) selective binding of a ligand (e.g., a monoclonal antibody, a polyclonal antibody, or functionally active fragments thereof containing an Fc domain) to a bacteriophage surface antigen to mediate targeting of the bacteriophage to cells that express an Fc receptor on their surface; and (4) modification of the bacteriophage genome to express a surface avidin-bacteriophage coat protein fusion product to which a biotinylated ligand (e.g., antibody) can be attached.

The simplest method for attaching a ligand to the surface of a bacteriophage is a chemical modification reaction in which the surface of the bacteriophage is subjected to galactosylation or lactosylation via N-linked glycosidic covalent linkages so as to attach galactose or lactose, respectively, to the bacteriophage surface. See, e.g., the Examples. Galactose- or lactose-labeled bacteriophages selectively bind to asialoglycoprotein receptors on the surface of hepatocytes to form a ligand-bacteriophage complex that is internalized by the target cell. Chemical modification also can be used to attach a peptide ligand to the bacteriophage surface. For example, peptide ligands containing a free amine group, carboxyl group, or sulfhydryl group can be attached to the bacteriophage surface using conventional procedures known to those of ordinary skill in the art for cross linking proteins. See, e.g., U.S. Patent No. 5,108,921, issued to Low et al. If the peptide ligand does not have a free amine or carboxyl group, such a group can be introduced by, for example, introducing a cysteine (containing a reactive thiol group) into the peptide ligand by site directed mutagenesis. Disulfide linkages can be formed between thiol groups in, for example, the peptide ligand and a protein expressed on the surface of the bacteriophage. For example, covalent linkages can be formed using bifunctional crosslinking agents that are known by those of ordinary skill in the art to have utility with respect to crosslinking peptides and proteins. Exemplary crosslinking agents include bismaleimido hexane

(which contains thiol-reactive maleimide groups and which forms covalent bonds with free thiols). See, also, the Pierce Co. Immunotechnology Catalogue and Handbook Vol. 1 for a list of exemplary homo- and hetero-bifunctional crosslinking agents, thiol-containing amines and other molecules with reactive groups for a comprehensive list of commercially available agents and corresponding peptide coupling chemistries that can be used to attach a peptide ligand to, for example, an amino acid functional group (e.g., amine) on the surface of a bacteriophage. Further exemplary coupling chemistries that are suitable for this purpose include methods which utilize the following crosslinking agents: glutaraldehyde (M. Riechlin, *Methods in Enzymology* 70:159-165 (1980); N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (T.L. Goodfriend, et al., *Science* 144:1344-1346 (1964); and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (M.H. Klapper and I.M. Klotz, *Methods in Enzymology* 25:531-536 (1972)). In general, the ligand-labeled bacteriophages can be prepared by using well-known methods for forming amide, ester or imino bonds between acid, aldehyde, hydroxy, amino, or hydrazo groups on the respective bacteriophage and ligand. In certain embodiments, reactive functional groups that are present in the amino acid side chains of the bacteriophage extracellular proteins or peptide ligands preferably are protected with a protecting group prior to coupling the ligand to the bacteriophage. As used herein, "protecting group" refers to a molecule which is bound to a functional group on a bacteriophage or peptide ligand and which may be selectively removed therefrom to expose the functional group in a reactive form. Preferably, the protecting groups are reversibly attached to the functional groups and can be removed therefrom using, for example, chemical or other cleavage methods. Thus, commercially available side-chain-blocked amino acids (e.g., Fmoc-derived amino acids from Advanced Chemtech, Inc., Louisville, KY) can be used to synthesize peptide ligands in accordance with standard peptide synthesis methods to obtain peptide ligands having side-chain-blocked amino acids. Alternatively, the peptide ligand side chains can be reacted with protecting groups after peptide ligand synthesis, but prior to the covalent coupling reaction. In this manner, the ligand-labeled bacteriophages of the invention can be prepared in which the amino acid side chains of the peptide ligand do not participate to any significant extent in the coupling of the peptide ligand to functional groups on the surface of the bacteriophage.

Alternatively, genetic engineering methods can be used to attach a ligand to the surface of a bacteriophage. For example, a sequence encoding the ligand ("ligand coding sequence") can be inserted, in frame, into the bacteriophage genome adjacent to or within a bacteriophage protein that is expressed on the bacteriophage surface to provide a fusion protein that contains

both functional ligand and a functional viral packaging protein. (See, e.g., the Examples.)

According to yet another embodiment, the ligand-labeled bacteriophage is formed by selectively binding an antibody or functionally active fragment thereof (i.e., an antibody fragment containing at least one antigen-binding site) to an antigen that is contained on the surface of the bacteriophage (e.g., a bacterial extracellular protein). The selectively bound antibodies mediate targeting of the bacteriophage to a target cell that contains on its surface an Fc receptor (e.g., a phagocyte or antigen presenting cell). Antibodies that are useful in accordance with this aspect of the invention are antibodies that exhibit a sufficiently high binding affinity for a bacteriophage antigen to result in little or no dissociation of the antibody-antigen complex under physiological conditions. In general, such antibody binding to the surface of the bacteriophage is performed by contacting the bacteriophage with an antibody that selectively binds to an antigen expressed on the bacteriophage surface under the same conditions that are used for performing an immunoassay, e.g., an ELISA, RIA. Exemplary conditions are described in Current Protocols in Immunology, ed. Coligan, J.E., et al., National Institutes of Health, John Wiley and Sons, Inc. (1994).

In a particularly preferred embodiment, the ligand is attached to the surface of the bacteriophage by means of an avidin/biotin complex. As used herein, "avidin" or "avidin peptide" refers to an avidin molecule, a streptavidin molecule, or a fragment or variant thereof that binds to biotin with an affinity that is approximately the same (i.e., within 10%) or greater than the affinity with which streptavidin binds to biotin. According to this embodiment, the bacteriophage is modified to express on its surface avidin or a portion thereof that selectively binds to biotin with the requisite binding affinity. Modification of the bacteriophage to express avidin is most easily accomplished by inserting the nucleic acid encoding avidin or a functionally active portion thereof into the bacteriophage genome such that the avidin or avidin portion is expressed on the bacteriophage surface. For example, the avidin can be inserted, in frame, into the D gene of the lambda coat protein, using well-defined restriction sites in the lambda phage. (See, e.g., the Examples and figures.) In this manner, an avidin-expressing bacteriophage is produced which serves as an intermediate for attachment of a biotinylated ligand to the bacteriophage surface.

Alternatively, avidin or a functionally active portion thereof can be chemically coupled to the bacteriophage surface using standard cross-linking chemistries, such as those described above. The avidin-labeled bacteriophage permits non-covalent, yet high affinity, attachment of

pre-selected biotinylated ligands to the bacteriophage surface for receptor-mediated targeted delivery to the mammalian target cell. Exemplary ligands which can be biotinylated in accordance with standard procedures are provided in Table 6. Alternatively, the bacteriophage can be biotinylated and an avidin-labeled ligand can be used to form the ligand-labeled bacteriophages described herein.

The bacteriophages of the invention are contacted with the target cell under conditions to permit selective binding of the ligand on the surface of the bacteriophage to the receptor on the surface of the target cell and to allow the bacteriophage to enter the target cell. Conditions which permit the binding of a receptor to its cognate ligand are the physiological conditions (e.g., the particular pH, ionic strength, viscosity) at which the ligands and receptors are known to bind to one another *in vivo* and the conditions at which the ligands and receptors are known to bind to one another *in vitro*, such as in receptor assays for determining the presence of a ligand in, for example, a biological fluid. Such conditions are known to those of ordinary skill in the art of receptor-mediated processes, such as receptor-based binding assays and receptor-mediated delivery of therapeutic agents to preselected tissues *in situ*.

In general, the conditions that allow the target cell to live and transcribe the therapeutic polynucleotide are the same conditions that permit selective binding of the ligand to the receptor and that allow the bacteriophage to enter the target cell. Optionally, the conditions that allow the cell to transcribe the therapeutic polynucleotide further include the addition of an inducer (see, e.g., Table 5) that activates an inducible promoter to induce transcription and translation of the therapeutic polynucleotide. The optimum conditions for inducing the transcription and translation of a therapeutic polynucleotide that is under the control of a particular inducible promoter can be determined by one of ordinary skill in the art using no more than routine experimentation. In general, for *in vitro* gene therapy, conventional tissue culture conditions and methods are used to sustain the mammalian cell in culture. For example, the mammalian cell can be allowed to live on a substrate containing collagen, e.g., type I collagen, or a matrix containing laminin, such as described in PCT application number PCT/US95/11456, having international publication number WO96/09074, entitled "Use of a Non-mammalian DNA Virus to Express an Exogenous Gene in a Mammalian Cell," and the references cited therein.

As used herein, "contacting", in reference to the bacteriophage and the target cell, refers to bringing the bacteriophage into sufficiently close proximity to the target cell to permit the receptor on the target cell to selectively bind to the ligand on the bacteriophage. Such conditions

are well known to those of ordinary skill in the art and are exemplified by the procedure provided in the Examples. See also, e.g., U.S. patent No. 5,108,921, issued to Low et al. which reports the conditions for receptor-mediated delivery of "exogenous molecules" such as peptides, proteins and nucleic acids to animal cells and U.S. patent No. 5,166,320, issued to Wu et al., which reports the conditions for the receptor mediated delivery of a ligand-gene conjugate to a mammalian cell. For a further discussion of the conditions and mechanisms by which receptor mediated delivery can be used to deliver an exogenous molecule into a target cell, and in particular, into a mammalian cell, see, e.g., S. Michael, et al., J.Biol., Chem. 268(10):6866 (1993), "Binding-incompetent Adenovirus Facilitates Molecular Conjugate-mediated Gene Transfer by the Receptor-mediated Endocytosis Pathway"; M. Barry, et al., Nature Medicine 2(3):299 (1996), "Toward cell-targeting gene therapy vectors: Selection of cell-binding peptides from random peptide-presenting phage libraries"; S. I. Michael, Gene Ther. 2:660 (1995), "Addition of a short peptide ligand to the adenovirus fiber protein".

The bacteriophage can be contacted with the targeted mammalian cell *in vitro*, for example, for *ex vivo* gene therapy or production of a catalytic RNA or recombinant protein in cell culture, or *in vivo* for *in vivo* gene therapy or *in vivo* production of a polynucleotide transcription or translation product. As used herein, a "mammalian target cell" refers to a mammalian cell (preferably, a human cell) which contains on its surface a receptor for the ligand that is contained (e.g., expressed) on the surface of the bacteriophage. Essentially, any mammalian cell can be targeted in accordance with the methods of the invention. The cell may be a primary cell or may be a cell of an established cell line. Exemplary cell types that can be targeted in accordance with the methods of the invention are provided in Table 4. Preferably, the mammalian cell is a hepatocyte (liver cell), a breast epithelial cell, a keratinocyte, a melanocyte, or a hematopoietic cell, e.g., erythrocyte, leukocyte, monocyte, or a lymphocyte. Screening methods, such as those described in the Examples, can be used to confirm that these and other target cells internalize the bacteriophage vectors of the invention via receptor-mediated endocytosis and, further, that these target cells express detectable levels of the exogenous polynucleotide insert. Such high-throughput screening methods can be used to select target cells that satisfy the above-noted criteria using no more than routine experimentation. In addition, such screening assays are predictive of receptor-mediated endocytosis of target cells *in vivo*.

Where the bacteriophage is contacted with the cell *in vitro*, the target cell subsequently can be introduced into the mammal (e.g., into the portal vein or into the spleen) if desired.

Accordingly, expression of the therapeutic polynucleotide is accomplished by allowing the cell to live or propagate *in vitro*, *in vivo*, or *in vitro* and *in vivo*, sequentially. Similarly, where the invention is used to express a therapeutic polynucleotide in more than one cell, a combination of *in vitro* and *in vivo* methods are used to introduce the therapeutic polynucleotide into more than one mammalian cell.

In *ex vivo* gene therapy, the cells are removed from a subject and a therapeutic polynucleotide is introduced (i.e., transfected) into the cells *in vitro*. Typically, the transfected cells are expanded in culture before being reimplanted into the mammalian recipient. The procedure for performing *ex vivo* gene therapy is outlined in U.S. Patent 5,399,346 and in exhibits submitted in the file history of that patent, all of which are publicly available documents. In general, *ex vivo* gene therapy involves the introduction *in vitro* of a functional copy of a gene into a cell(s) of a subject which contains a defective copy of the gene, and returning the genetically engineered cell(s) to the subject. The functional copy of the gene is under the operable control of regulatory elements which permit expression of the gene in the genetically engineered cell(s). In *in vivo* gene therapy, the target cells are not removed from the patient. Rather, the therapeutic polynucleotide is introduced into the cells of the mammalian recipient *in situ*, i.e., within the recipient. In general, the improved gene therapy method disclosed herein is practiced by using the ligand-labeled bacteriophage of the invention in place of the gene therapy vectors disclosed in the prior art (e.g., adenoviral vectors, modified adenoviral vectors, retroviral vectors, plasmids, liposomes) in the procedures currently used for administering these vectors (or cells containing these vectors) to the subjects. Such procedures are known to those of skill in the art of human gene therapy. (See, also, the references identified below with respect to *in vivo* gene therapy.)

A particularly preferred embodiment of the invention is illustrated in the Examples, which describes the construction of a lambda bacteriophage for use as a gene therapy vector. Briefly, a bacteriophage containing an exogenous polynucleotide (encoding beta-galactosidase) was chemically modified (galactosylated) to contain galactose residues on the bacteriophage surface. The galactose-labeled bacteriophage was internalized by HepG2 cells (a hepatoma cell line) in culture, presumably, by binding of the labeled bacteriophage to the asialoglycoprotein receptor present on the surface of the hepatocytes.

Insertion of the therapeutic polynucleotide into the target cell genome may be either transient or permanent. By "transient", it is meant that the bacteriophage genome lacks the

capacity to replicate and/or to segregate to progeny cells. For example, the therapeutic polynucleotide may be epigenetic and without the capacity to replicate and segregate to progeny cells (e.g., lacks an origin of replication, appropriate telomere and centromere structures). "Transient" insertion into the target cell also occurs, for example, when the bacteriophage is used to infect cells of limited replicative capacity, i.e., non-stem cells. "Permanent" insertion of the therapeutic polynucleotide into the target cell is accomplished by, for example, (1) infecting stem cells that produce bacteriophage-bearing progeny; or (2) including recombination sequences in the bacteriophage genome on either side of the therapeutic polynucleotide so as to promote reasonably efficient homologous recombination of the therapeutic polynucleotide into a defined sequence of the target cell genome or (3) random integration into the host cell chromosomal DNA. "Permanent" insertion also can be achieved by including in the bacteriophage an origin of replication, telomeres and centromeres to obtain a bacteriophage that autonomously replicates (i.e., an "artificial chromosome") and is capable of segregating into progeny cells. If the bacteriophage genome is autonomously replicating, it is preferred to further include in the bacteriophage genome appropriate enhancer-promoter sequences, such as those described in the aforementioned tables.

For *in vivo* gene therapy, the bacteriophage is administered to the mammalian recipient, for example, intravascularly, intraluminally (introduction of the bacteriophage into body cavities and lumens, such as the genital urinary tract, gastrointestinal tract, trachea-bronchopulmonary tree or other internal tubular structures), direct injection into a tissue (e.g., muscle, liver), topical application (e.g., eye drops or aerosol application to mucosal surfaces), or intracavitary (e.g., intraperitoneally or intrathecally (introduction into the cerebrospinal fluid). Optionally, an implantable pump or other device or implant (preferably, a bioerodible implant) to effect the sustained release of the bacteriophage can be used to facilitate delivery of the bacteriophage to the mammalian targeted cell over a pre-selected period of time (e.g., sustained release over a period of days to sustained release over a period of weeks to months). Although the ligand/receptor-mediated delivery of the bacteriophage is the predominant mechanism for targeting delivery of the bacteriophage to a particular cell type, delivery to the target cell can further be modulated by regulating the amount of bacteriophage administered to the mammalian recipient and/or by controlling the method of delivery. Thus, for example, intravascular administration of the bacteriophage to the portal vein or to the hepatic artery can be used to facilitate targeting the bacteriophage to a liver cell.

In general, the bacteriophage can be administered to the mammalian recipient using the same modes of administration that currently are used for adenovirus-mediated gene therapy in humans. Such conditions are adequate for contacting the bacteriophage and the target cell under conditions to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and to allow the bacteriophage to enter the target cell. These conditions are described in the following references: PNAS 90:10613 (1993); Nature Medicine 1:1148 (1995); Nature Medicine 12:266 (1996); New Engl. J. Med. 333:832 (1995); and New Engl. J. Med. 333:823 (1995). Preferably, the bacteriophage is administered to the mammalian recipient by intravascular injection, intra-organ introduction by, for example, injection into the organ or contacting the bacteriophage with the organ in the presence of a tissue permeabilizing agent; and introduction of the bacteriophage into body cavities or lumens. Optionally, immunosuppressive drugs, such as glucocorticosteroids or cyclophosphamide are co-administered with the bacteriophage to suppress a primary immune response that may be triggered by an initial exposure to a foreign antigen. Mammalian cells which have been transfected with the bacteriophage *ex vivo* can be introduced into the mammalian recipient using the known methods for implanting transfected cells into a human for gene therapy. See, e.g., U.S. Patent No. 5,399,346 ("Gene Therapy") issued to Anderson et al.; PCT International application no. PCT/US92/01890 (Publication No. WO 92/15676, "Somatic Cell Gene Therapy", claiming priority to U.S. Serial No. 667,169, filed March 8, 1991, inventor I. M. Verma); PCT International application no. PCT/US89/05575 (Publication No. WO 90/06997, "Genetically Engineered Endothelial Cells and Use Thereof", claiming priority to U.S. Serial No. 283,586, filed December 8, 1989, inventors Anderson, W.F. et al.).

The invention is not limited in utility to human gene therapy, but also can be used in the manufacture of a wide variety of proteins and nucleic acids that are useful in the fields of biology and medicine. The bacteriophages of the invention advantageously provide a method for synthesizing gene products from genes which range in size from about one to one-hundred kilobases. Further, the invention provides a method for providing the bacteriophage vectors at a low cost, namely, by propagating the bacteriophages in a prokaryotic host. Moreover, the invention provides a simple method for preparing mammalian proteins, including proper post-translational modifications, *in vitro*. Thus, the invention provides an improved method for introducing an exogenous polynucleotide into a mammalian cell. The improved method involves contacting the bacteriophages of the invention (which contain the exogenous polynucleotide)

with the mammalian cell and allowing the bacteriophage to enter the cell, gain access to the nucleus, and replicate the exogenous polynucleotide therein. As discussed above, the bacteriophage contains on its surface the ligand that selectively binds to a receptor for the ligand that is contained on the surface of the mammalian cell.

As used herein, an "exogenous polynucleotide" refers to a nucleic acid that is not normally present in the bacteriophage genome and that is inserted into the bacteriophage using recombinant engineering methodology. Examples of exogenous polynucleotides include the above described therapeutic polynucleotides, as well as regulatory polynucleotides which are not intended for therapeutic applications (e.g., polynucleotides that are introduced into the mammalian cell *in vitro* or *in vivo* for the purpose of producing a mammalian protein/protein complex *in vitro* or *in vivo*).

According to yet another aspect of the invention, an avidin-labeled bacteriophage that is used as an intermediate in connection with the above described methods for introducing a therapeutic or other exogenous polynucleotide into a mammalian cell is provided. The intermediate can be used to prepare a bacteriophage having on its surface virtually any ligand, provided, that the ligand can be biotinylated and retain its binding activity to a receptor. Thus, the invention also provides a method for preparing a ligand-labeled bacteriophage which involves contacting an avidin-expressing bacteriophage with a biotinylated ligand under conditions to permit binding of the avidin to the biotin. As will be apparent to those of ordinary skill in the art, alternative binding pairs can be used in place of the avidin-ligand binding pairs to accomplish this same objective. Such binding pairs include, for example, streptavidin-biotin binding pairs, antibody antigen, and any other high affinity interactions. Thus, the invention provides a generic mechanism for forming a bacteriophage which contains on its surface virtually any type of ligand. The avidin-labeled bacteriophage can be provided as a component of a kit for labeling a bacteriophage with a ligand of choice. The kit includes instructions for forming a ligand-labeled bacteriophage by allowing the avidin-bacteriophage to react with a biotinylated ligand under conditions to permit selective binding of the avidin-labeled bacteriophage to the biotinylated ligand. Optionally, the kit further includes reagents, and appropriate instructions, for biotinylating a ligand of choice.

The invention provides other compositions and kits which are useful for practicing the above-described methods. According to a particularly preferred aspect of the invention, a bacteriophage of the invention is provided. The bacteriophage contains (a) a bacteriophage

genome containing an exogenous polynucleotide that can be transcribed in a mammalian cell; and (b) a ligand contained on the surface of the bacteriophage that selectively binds to a receptor expressed on the surface of a mammalian cell. The bacteriophages of the invention optionally are contained in a pharmaceutically acceptable carrier to form a pharmaceutical composition.

5 The pharmaceutical compositions should be sterile and contain a therapeutically effective amount of the bacteriophages (or target cells containing the bacteriophages) in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is
10 compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

In one particular embodiment, the preferred pharmaceutical composition is contained in
15 an implant that is suitable for implantation into the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. PCT/US/03307 (Publication No. WO 95/24929, entitled "Polymeric Gene Delivery System", claiming priority to U.S. patent application serial no. 213,668, filed March 15, 1994). PCT/US/0307 describes a biocompatible, preferably biodegradable polymeric matrix for
20 containing an exogenous gene under the control of an appropriate promoter. The polymeric matrix is used to achieve sustained release of the exogenous gene in the patient. In accordance with the instant invention, the bacteriophage particles described herein are encapsulated or dispersed within the biocompatible, preferably biodegradable polymeric matrix disclosed in PCT/US/03307. The polymeric matrix preferably is in the form of a micro particle such as a
25 micro sphere (wherein the bacteriophage particle is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein the bacteriophage particle is stored in the core of a polymeric shell). Other forms of the polymeric matrix for containing the bacteriophage particle include films, coatings, gels, implants, and stents. The size and composition of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix device is implanted. The size of the polymeric matrix further is selected according to the method of
30 delivery which is to be used, typically injection into a tissue or administration of a suspension by aerosol into the nasal and/or pulmonary areas. The polymeric matrix composition can be

selected to have both favorable degradation rates and also to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when the polymeric matrix is administered to a mucosal surface. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver the bacteriophage particles of the invention to the subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. Synthetic polymers are preferred. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multi-valent ions or other polymers.

In general, the bacteriophage particles of the invention are delivered using the bioerodible implant by way of diffusion, or more preferably, by degradation of the polymeric matrix.

Exemplary synthetic polymers which can be used to form the biodegradable delivery system include: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terphthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, poly-vinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terphthalate), poly(vinyl alcohols), polyvinyl acetate, polyvinyl chloride, polystyrene and polyvinylpyrrolidone.

Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof.

Examples of biodegradable polymers include synthetic polymers such as polymers of

lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(butic acid), poly(valeric acid), and poly(lactide-cocaprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water *in vivo*, by surface or bulk erosion.

Bioadhesive polymers of particular interest include bioerodible hydrogels described by H.S. Sawhney, C.P. Pathak and J.A. Hubell in Macromolecules, 1993, 26, 581-587, the teachings of which are incorporated herein, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate). Thus, the invention provides a composition of the above-described bacteriophages for use as a medicament, methods for preparing the medicament and methods for the sustained release of the medicament *in vivo*. In the preferred embodiments, the bacteriophage is selected from the group of bacteriophages shown in Table 1 and is labeled with an appropriate ligand as described above. Preferably, the bacteriophage is selected from the group consisting of a lambda phage, a P1 phage, a P22 phage, and an SPP1 phage; more preferably, the bacteriophage is a lambda phage or a P1 phage; most preferably, the bacteriophage is a lambda phage.

Examples

Introduction to the Examples

In the present method, a bacteriophage particle serves as a vector for gene delivery to a mammalian cell. The bacteriophage capsid and/or tail are modified to contain a ligand (or an adapter, e.g., avidin, for a ligand) that can bind to a receptor contained on the surface of a mammalian cell and facilitate entry therein. Following receptor-ligand interaction the recombinant bacteriophage can be internalized via receptor mediated endocytosis or other receptor-mediated mechanisms of intracellular transport. The bacteriophage genome is engineered to include transcriptional control signals, which allow expression of an exogenous polynucleotide in the mammalian cell. Optionally, the recombinant bacteriophage further contains an endosomal lysis signal, such as that contained within the capsid-stabilizing protein VI and L3/p23 of adenovirus (Greber UF et al. EMBO J, 1996; 15: 1766) to promote export of the bacteriophage genome into the cytoplasm prior to fusion of the endocytosed vesicles with mammalian cell lysosomes and enzymatic degradation of the bacteriophage genome. Conventional recombinant DNA techniques can be used for inserting these and other nucleic acid sequences into the phage genome. For example, the adenovirus endosomal lysis signal can be inserted in-frame into or appended to the bacteriophage tail fiber J gene.

To effect successful transduction after entry of the recombinant bacteriophage into the cytoplasm, the bacteriophage genome is delivered to the nucleus. In dividing cells, the genome of the bacteriophage used in the invention is efficiently delivered to the nucleus during mitosis when the nuclear membrane dissipates and reforms. For delivery of the bacteriophage genome to the nucleus of non-dividing cells, the phage particle is modified to include nuclear localization signals (NLS) that mediate transport of molecules or macromolecular aggregates, such as viral DNA across the nuclear membrane and into the nucleoplasm. Such nuclear localization signals are known to those of ordinary skill in the art and include portions of the human immunodeficiency virus (HIV) proteins matrix (MA) and Vpr (Naldini L et al. Science 1996; 272: 263; Bukrinsky MI et al., Nature 1993; 365: 666), large T antigen of SV40 (Kalderon D et al. Cell 1984; 39: 499; *Drosophila* Antennapedia (Derossi D et al. J Biol Chem 1996; 271: 18188), VP22 of Herpes virus (Elliott G. et al., Cell 1997; 88: 223), *X. laevis* nucleoplasmin, *X. laevis* lamin L, human c-myc encoded protein, and adenovirus type 2/5 Ela (Chelsky D et al. Mol

Cell Biol 1989; 9: 2487). For example, the HIV or SV40 nuclear localization signal can be inserted in frame into or appended to the tail fiber J protein or the H protein which is thought to bind tightly to the bacteriophage genome. Exemplary nuclear localization signals as disclosed in the above-identified references are provided in SEQ. ID NOS. 7-14.

5 In a preferred embodiment, the bacteriophage genome further includes adeno-associated viral sequences that mediate integration into specific chromosomal regions of the mammalian cell to effect delivery of the transduced bacteriophage genome to a specific mammalian cell chromosomal location. Such adeno-associated viral sequences are known to those of ordinary skill in the art. In yet other embodiments, the bacteriophage has a genome of sufficient size (e.g.,
10 P1 phage) to permit the further inclusion of nucleic acid sequences located 5' and 3' of the exogenous polynucleotide to promote site directed homologous recombination. More preferably, the bacteriophage has a genome of sufficient size (e.g., P1 phage) to permit insertion of a large genetic unit containing not only one or more coding sequences of interest and their associated control regions, but in addition, mammalian autonomous replicating sequences, as well as
15 centromere and telomere sequences in a self replicating format to form, in effect, an artificial chromosome. The ability to deliver exogenous genetic material of large size (e.g., a complete gene, including regulatory sequences) has obvious advantages with respect to achieving stable and tissue specific expression *in vivo* and *in vitro*.

A description of several bacteriophage vectors that can be used in accordance with the
20 methods of the invention follows. Briefly, Example I illustrates the construction of three bacteriophage λ vectors containing exogenous genetic material; Example II illustrates the post-packaging modification of bacteriophage λ capsid and tail proteins to form a galactose-labeled bacteriophage λ ; Example III illustrates the targeted delivery of a modified bacteriophage of the invention to human hepatoma G2 (HEP G2) cells grown *in vitro*; Example IV illustrates the
25 targeted delivery of a modified bacteriophage of the invention to hepatocytes in the liver of live mice *in vivo*; Example V illustrates the construction of bacteriophage vectors containing modified tail proteins; and Example VI illustrates screening methods for selecting bacteriophages and target cells that transduce mammalian cells via receptor-mediated endocytosis. These examples are provided for illustrative purposes only, and their inclusion is not meant to limit the
30 scope of invention. Thus, each of the procedures described in reference to the bacteriophage λ can be performed using any of the bacteriophages identified in Table 1 by: (1) obtaining the

published restriction maps for these bacteriophages; (2) selecting an exogenous polynucleotide (e.g., a gene having a published sequence that encodes a therapeutic polynucleotide product, such as identified in Tables 2 or 3); (3) inserting the gene into the well-defined restriction sites of the bacteriophage genome using substantially the same procedures described herein (e.g., blunt-ended ligation into bacteriophage lambda) or alternative procedures known to one skilled in the art for the insertion of a polynucleotide into a restriction site of a bacteriophage; (4) modifying the surface of the bacteriophage to include a ligand that targets delivery of the modified bacteriophage to a mammalian cell that contains on its surface the cognate receptor for the ligand (such as identified in Table 6) using substantially the same procedures described herein (e.g., galactosylation of the bacteriophage lambda phage particles, otherwise chemically modifying the surface of the bacteriophage to attach a ligand, or engineering the bacteriophage to express the ligand as a surface protein or polypeptide); and (5) contacting the modified bacteriophage with the target cell under conditions that permit the selective binding of the bacteriophage ligand to the cognate receptor using substantially the same conditions described herein for *ex vivo* and *in vivo* targeted delivery of bacteriophage lambda or alternative procedures known to one of ordinary skill in the art. By "substantially the same conditions", it is meant that the conditions are modified to substitute a different bacteriophage for bacteriophage lambda in the procedure and to use the published restriction enzymes, buffers, incubation times, size inserts and so forth that are known to be appropriate for modifying the different bacteriophage to include an exogenous polynucleotide and contain a surface ligand.

Example I Construction of three bacteriophage λ vectors

(a) Construction of the CMV- β -gal - lambda DASH II Chimeric DNA Sequences

The CMV β -gal I chimeric DNA sequences used in these experiments was constructed as follows. A 10.7 kilobase pair (kb) Eco RI fragment excised from plasmid adCMV/ NLS β -gal (obtained from Dr. Ronald Crystal, Columbia University; see Figure 3) that contained the reporter gene expression cassette was ligated to Eco RI/ BamHI double-digested left and right arm of λ DASH II (Stratagene, La Jolla, CA), a derivative of λ 2001, which contains an extended range of cloning sites. The genetic and physical map information used in this invention was obtained from published wild type λ DNA sequences and from the GenBank data base (accession number J02459).

The 10.7 kb reporter gene expression cassette included DNA sequences corresponding to

the CMV promoter/ enhancer linked to the E. coli β -gal gene. The expression cassette also included sequences encoding simian virus 40 (SV40) RNA splicing donor / acceptor sites and polyadenylation signals.

5 Generation of recombinant λ phage particles. CMV β -gal containing recombinant λ phage particles were generated by packaging the ligated CMV β -gal λ DASH II chimeric DNA with an *in vitro* packaging extract, Giga pak Gold II (Stratagene, La Jolla, CA), according to the manufacturer instructions.

10 Large scale preparation of recombinant phage particles. Conventional methods can be used to propagate the recombinant λ phage (e.g., Meth. Enzymol. 152:145-170, 1987). For example, the bacteriophage λ that was used in this experiment was grown and amplified from a single agar plug. Liquid lysates were made by growing the E. coli strain XL1 blue MR (P2) (genotype Δ (mcr A)) 183 Δ (mcr CB- hst MR) 173 end A 1 sup E 44 thi-1 gyr 96 rel A1 lac (P2 lysogen), to
15 A550 greater than or equal to 0.5 in Luria broth containing 5mM CaCl₂ at 37°C with vigorous shaking. At this point, the phages were added to a "moi" (multiplicity of infection) of 0.01. After 4-5 hrs of vigorous shaking, the lysed culture broth was centrifuged to remove cell debris. Amplified bacteriophages were concentrated by polyethylene glycol (PEG) precipitation from the lysate in accordance with standard procedures. PEG precipitated phages were further
20 purified by CsCl₂ gradient centrifugation in accordance with standard procedures. (See, e.g., Meth. Enzymol. 152:145-170, 1987 for standard PEG precipitation and CsCl₂ gradient purification protocols.)

25 Construction of phosphoglycerokinase (PGK) β -gal λ DASH II chimeric DNA sequences.

Expression from the CMV promoter/enhancer by mouse liver *in vivo* reportedly is extremely low (Furth, A., et al., Nucleic Acid Research 19:6205-08 (1991)). Accordingly, for *in vivo* targeted delivery of reporter β -gal gene to mouse liver, a chimeric PGK (phosphoglycerokinase) promoter/enhancer/ β -gal/ λ DASH II (Fig. 4) was constructed as follows. Eco RI and Bal I digested 0.6 kb fragment released from the PGK promoter containing plasmid (Gene, 80:65,
30 1987) was subcloned into Eco RI Xho I/ blunt site of adaCMV/ NLS/ beta-gal plasmid. The resulting plasmid (designated PGK-NLS- β -gal) was Eco RI digested and ligated to λ DASH II

sequences. After ligation, the chimeric PGK-NLS- β -gal λ DASH II DNA was packaged *in vitro* and subsequently propagated in *E. coli* for large scale production of phage particles.

Construction of MCK-DMD- λ DASH II chimeric DNA sequence. As discussed previously, a significant advantage of the bacteriophage λ used in this invention is that it can be readily engineered to permit large gene-containing expression cassettes that could never be achieved by viral vectors currently used for delivery to mammalian cells. An example is a phage vector containing the MCK-DMD gene, which at 20.3 kb (Fig 5) greatly exceeds the capacity of currently used vectors. This vector was constructed as follows. The 20.3 kb expression cassette containing muscle creatine kinase (MCK) promoter/enhancer 5' to the full length DMD cDNA (obtained from J. Chamberlain, U. Michigan) was released from the plasmid pMDA after digestion with BssHII enzyme in accordance with standard procedures. This fragment was blunt ended and ligated to λ DASH II DNA sequences. The MCK-DMD gene / λ DASH II DNA sequences were used for generating the recombinant bacteriophage λ particles after packaging the chimeric DNA sequences with *in vitro* packaging extract (as described above).

Construction of λ phage particles displaying the ligand "L" heregulin for targeted gene delivery

Construction of λ phage particle that displays a specific ligand can be used for delivering the gene to a specific cell or tissue expressing the corresponding receptor via receptor/ligand mediated endocytosis. DNA sequences that encode ligand(s) known to be endocytosed after interaction with cognate receptor(s) were inserted, in frame, into the D-gene locus of the λ phage genome to produce a transcription template for a chimeric D gene-ligand bifunctional protein that expresses the fusion protein (including the ligand(s) of choice) on the bacteriophage surface. A similar strategy recently has been reported for targeting retrovirus vectors for gene delivery into a specific cell or tissue type (Proc. Natl. Acad. Sci. 92:9747-51 (1995)). For example, ligand directed retroviral targeting of human breast cancer cells recently has been reported by constructing retrovirus vectors expressing heregulin -gp70 chimeric envelope genes.

Alternatively, ligand directed λ phage targeting of specific mammalian cells is achieved by expressing chimeric λ D-gene and immunoglobulin single chain variable fragments (SCVF) directed against receptors such as LDL and IL-2, both of which are known to be internalized after the antibody-receptor interaction. Recently this approach has been used to generate

retrovirus vectors expressing chimeric envelope and SCVF for LDL receptor protein to transfer a β -gal gene to human cells expressing the LDL receptor (Proc. Natl. Acad. Sci. 92:7570).

Bacteriophage λ particles having a capsid that displays a chimeric λ D-gene-avidin fusion protein also can be used to effect targeted delivery of the phage particles to a specific mammalian cell. Since the avidin molecule has very high affinity for biotin, any peptide or polypeptide ligand that can be biotinylated without adversely affecting the ability of the receptor to mediate endocytosis can be used as described herein. The avidin gene, or portion thereof encoding the polypeptide that selectively binds to biotin, is inserted, in frame, into the bacteriophage genome using the procedures described herein for forming a chimeric λ D-gene and the published cDNA nucleic acid sequence for avidin (Gope, L. Mohan, et al., Nucleic Acid Research 15:3595-3606 (1987) and GenBank Accession No. 451889).

Fig. 6 illustrates the construction of a λ phage particle expressing the heregulin-D-gene chimeric DNA sequences on its capsid. In this construction, the D gene heregulin sequences were first fused by generating 3 fragments (AB, CD, and EF) by polymerase chain reaction (PCR) in accordance with standard procedures using Taq polymerase and 6 sets of primers (a,b,c,d,e,f):

primer a (SEQ. ID NO. 1) ATACCGAGGGCTGCAGTGTACA

primer b (SEQ. ID NO. 2) CTCTTTCAATTGGGGAGGCAAAACGATGCTGATTGCCGTTC

primer c (SEQ. ID NO. 3) TTGCCTCCCCAATTGAAAGAG

primer d (SEQ. ID NO. 4)

GTGATGAAGGGTAAAGTTATTTGCGTTTTTTTTTCGGCGGGGTCCTCCATAAATT
CAATC

primer e (SEQ. ID NO. 5)

TAACTTTACCCTTCATCACTAAAGGCC

primer f (SEQ. ID NO. 6)

AAACGTACAGCGCCATGTTTACCAG

Gel purified fragments AB, CD and EF were treated with T4 polymerase to remove the overhanging nucleotide "A" in the fragment generated by terminal transferase activity present in the Taq polymerase used for PCR amplification. This step ensures the joining "in frame" of the coding sequences of both the λ D-gene and heregulin. Next,

fragments AB and CD were joined by first denaturing and then annealing the partial overlapping sequences, followed by extension with Taq polymerase, and then amplification after the addition of primers a and d. The fragment AD was gel purified, treated with T4 polymerase, and subsequently used for joining fragment EF (as described above). The fused λ D-gene/hereregulin gene containing fragment AF was subcloned into the TA cloning vector (Invitrogen, San Diego, CA). The clones containing the AF fragment were identified by determining the sequences with a double-stranded sequencing method using the Sequenase 2.0 kit (USB, Cleveland, OH). The AF fragment was generated after digestion of the TA plasmid clone with the restriction enzyme BsrGI and ligated into the necessary fragments of λ (as shown in Fig. 6). This chimeric DNA construct was used to generate λ phage particles having a genome that includes the recombinant D-gene/hereregulin construct and a capsid that displays this chimeric protein.

Example II

Post-packaging modification of bacteriophage λ capsid and tail proteins to form a galactose-labeled bacteriophage λ

Chemical Modification.

The particular procedure for modifying the bacteriophage λ capsid and tail proteins to form a galactose-labeled bacteriophage λ is described herein. The galactose-labeled bacteriophage particles can be used to target liver cells which express a unique asialo-glycoprotein receptor. This procedure is based upon the published procedures for forming an artificial asialo-glycoprotein containing lactose (Neda, H., et al., JBC 296:14143-14146 (1991)) or galactose (Human Gene Therapy 5:429-435 (1994)).

Method. CsCl₂ purified CMV β -gal reporter gene containing λ phage particles (10^{11} PFU/ml) were galactosylated in 2 ml reaction volume containing 60mg of galactose and 100 mg of 1 ethyl-3-(3-diethylaminopropyl) carbodiiminide (EDC, Sigma Chemical Corp., Saint Louis, MO) in sodium chloride solution. The pH of the unbuffered solution was adjusted to 7.5 with NaOH and the reaction mixture was incubated at room temperature for various lengths of time ranging from 24 to 48hrs. The galactose associated with phage particles was determined using ¹⁴[C]-labeled galactose. Twenty five μ l of ¹⁴[C]-galactose (250-360mCi/mM, DuPont NEN, Boston, MA) were included

in a 100:1 reaction mixture except the cold galactose was omitted. At the end of the different incubation periods, the solutions were filtered and washed through nitrocellulose filters in a vacuum filtration device. The filters were removed and counted in a Beckman scintillation counter (Palo Alto, CA). The radioactivity on filters was converted to numbers of 14 [C]-galactose on the basis of the specific activity. The extent of galactosylation was expressed as numbers of galactose/phage particles.

As described in the detailed description of the invention, a variety of chemical methods can be used to attach a ligand to bacteriophage capsid and/or tail proteins. For example, small peptide ligands, such as insulin, epidermal growth factor (EGF), keratinocyte growth factor (KGF), Fab fragments for anti-polymeric immunoglobulin receptors can be covalently linked to λ phage using the hetero-bifunctional crosslinking reagent N-succinimidyl 3-(2-pyridyl dithio) propionate (SPDP) or other bifunctional crosslinking agents in accordance with manufacturer's instructions. Additional crosslinking agents are provided in the description and are known to those of ordinary skill in the art. After coupling the ligand to the bacteriophage surface proteins, the modified bacteriophage is used to selectively deliver the gene of interest to target cells that express a cognate receptor for the ligand (Example III).

Example III

Targeted delivery of a Modified Bacteriophage of the Invention to human hepatoma G2 (HEP G2) cells grown *in vitro*

In vitro targeted delivery of CMV β -gal expression cassette containing galactose modified λ phage particles.

1. Growth of cells:

Conventional tissue culture methods were used to grow HEP G2 cells. HEP G2 cells were cultured in minimal essential medium as modified by Eagle (EMEM) containing 10% FBS. Cells were seeded one day prior to the addition of λ phage particles for gene transfer experiments. *In vitro* targeted delivery of the β -gal gene to HEP G2 cells was accomplished by allowing the phage particles to interact with the cells in tissue culture growth medium for about 6-10 hrs; more preferably, for 8-10hrs. In general, after galactosylation, 10^7 - 10^{10} total phage particles/ml for 6-10 hrs, preferably 10^7 particles/ml for 10 hours, are needed for efficient transduction of targeted cells. After

exposing the cells to phage particles for the appropriate time, the phage-containing medium was removed and replaced with fresh media.

2. Detection of internalization of bacteriophage vectors and gene expression:

After ligand-receptor mediated endocytosis of a bacteriophage vector into a mammalian cell, the expression of the exogenous genetic material in the mammalian cell can be monitored using standard methodologies. For example, delivery of a bacteriophage modified with a CMV β -gal recombinant construct and a galactose ligand to a HEP G2 cell receptor can be measured by detecting bacteriophage DNA or RNA by Southern or northern blotting or *in situ* hybridization with or without amplification by polymerase chain reaction (PCR). Radioactively-labeled DNA or RNA probes that selectively hybridize to unique portions of the phage genome, e.g., portions corresponding to a regulatory sequence, such as a promoter, or portions corresponding to the exogenous genetic material, such as the reporter gene β -galactosidase, can be designed and constructed using standard molecular biology techniques. Where the modified bacteriophage is to be used for delivering exogenous genetic material to a mammalian cell *in vivo* (e.g., to hepatocytes in murine liver), delivery of the phage to the specific cell can be detected by obtaining the targeted cells in a biopsy and assaying the biopsied cells using, for example, the above-mentioned conventional methods (e.g., Southern or northern blotting).

In general, expression of a gene of interest (e.g., therapeutic or marker polynucleotide) in a mammalian cell is detected by measuring the functional or immunological activity of the expressed gene in the targeted tissue, targeted cell, or body fluid (e.g., serum, lymph fluid). Alternatively, direct RNA or protein analysis for detecting specific transcription or translation products can be performed in accordance with standard practice. Exemplary detection techniques to measure gene expression include one or more of the following techniques, alone or in combination: northern or western blotting, *in situ* hybridization, reverse transcription, PCR amplification, immunostaining, RIA and ELISA. Such routine techniques also can be used to measure the stability of expression and maintenance of the delivered exogenous genetic material in the mammalian target cell by, for example, measuring the expression of one or more

reporter (e.g., marker) genes as a function of time following transduction. In an exemplary protocol, a reporter gene #1 initially is delivered to the target mammalian cells. One week later, reporter gene #1 expression is assayed. One to three months following the initial transduction, a reporter gene #2 is transduced into the same population of target cells. One week later, the ratio of reporter gene #1 to reporter gene #2 is determined by, for example, immunostaining (e.g., using a different dye for each immunohistologic assay) the target tissue to assess the stability and maintenance of expression of the exogenous genetic material in the target cell population. A diminution of reporter gene #1 expression compared to reporter gene #2 expression can indicate either immune attack against transduced cells or transduction of short-lived, differentiated cells. An exemplary procedure for processing a target tissue to which a β -galactosidase reporter gene expression has been delivered to determine whether the reported gene is expressed therein includes: a) snap freezing the tissue in isopentane chilled with liquid nitrogen, b) mounting the tissue on cryomold (Tissue -Teck, Miles, Elkhart, IN) using OCT and freezing, c) cutting the frozen tissue with a microtome at -20°C into 10 μm sections, d) staining the tissue for β -galactosidase reporter gene expression with X-gal (1mg/ml) (a. reporter gene substrate, Boehringer Mannheim, Indianapolis, IN), potassium ferro- and ferricyanide (35mM each) in phosphate buffered saline solution. To detect expression of a therapeutic polynucleotide, the above-described procedure is used with the exception that immunostaining using an antibody to detect the therapeutic polynucleotide expression product is used in place of the reporter gene substrate assay.

In the following example, delivery of the CMV β -gal gene-containing galactosylated and ungalactosylated phage particles *in vitro* to HepG2 cells and *in vivo* (Example IV) to liver cells of mice was measured. Histochemical staining of HepG2 cells using X-gal was used to measure expression of the β -gal reporter gene. The results showed a field of Hep G2 cells expressing the β -gal gene, as indicated by the appearance of positive cells that stained darkly following incubation with the substrate X-gal. The control experiment (ungalactosylated phage particles) did not show positive cells that stained darkly following incubation with the substrate X-gal. These results demonstrate that the galactose-labeled phage particles were selectively internalized by the murine liver cells, presumably via receptor-mediated endocytosis.

Example IV**The targeted delivery of a modified bacteriophage of the invention to hepatocytes in the liver of live mice *in vivo*.**

For the *in vivo* experiment, 10^{10} phage particles, either modified with an average of 50 galactose residues/phage particle or unmodified (control), were injected into mice intraperitoneally. Three days after injection, mice were sacrificed and liver and kidney tissue biopsies were prepared. Frozen sections were cut and fixed with 1% glutaraldehyde and subsequently stained with X-gal. After staining, tissue sections were analyzed by light microscopy. Unmodified phage (control) injection did not result in β -gal expression in either liver or kidney tissue sections that were stained with X-gal as detailed above. In contrast, injection of galactosylated phage resulted in detectable β -gal staining in hepatocytes but not in any cells on kidney tissue sections from the same mouse. Careful analysis of 40 serial sections of liver removed from the mouse exposed to the galactosylated phage demonstrated that approximately 0.5 to 1% of hepatocytes stained with X-gal and thus, effectively were transduced by the recombinant bacteriophage vector. While this example shows that intraperitoneal administration could be used successfully to transduce hepatocytes *in vivo*, one skilled in the art reasonably would believe that alternative routes of administration (such as those described in the detailed description), as well as the further inclusion of endosomal lysis signals and/or nuclear localization signals also can be used to successfully transduce mammalian target cells *in vivo* and *in vitro*.

Example V**Construction of Bacteriophage Lambda vectors with Modified Tail Proteins.**

As described above, bacteriophage with wild type packaging proteins and the beta-galactosidase gene are not effective genetic vectors for transducing Hep G2 cells. However, chemical modification of the surface proteins *in vitro* with galactose such that it will bind to and be internalized by the galactose-receptor on the Hep G2 cell surface does result in an effective vector. The galactose-asialoglycoprotein ligand-receptor interaction and subsequent internalization via receptor mediated endocytosis have been described in detail in the literature. Accordingly, the above-noted results, together with the literature reports documenting the mechanism of asialoglycoprotein-mediated transport, supports our hypothesis that the transfer of genetic material from the above-described modified bacteriophages to a mammalian cell target occurs via receptor-mediated endocytosis.

Thus, we believe that the mechanism by which modified bacteriophage vectors transfer genetic material to mammalian target cells is fundamentally different from the mechanism by which wild type bacteriophages transduce their natural prokaryotic hosts and that functional bacteriophage tail proteins are not essential for mammalian target cell transduction using the bacteriophages of the invention. This hypothesis is confirmed using the screening methods described below.

Using standard methods, endocytosis inhibition agents (e.g., colchicine, cytochalasin B and D, and monodansylcadaverine) are used to arrest cell-mediated endocytosis in a mammalian cell and, thereby, select for bacteriophages whose mechanism of transfer does not depend on injection of genetic material into the mammalian cell as is observed in the naturally occurring bacteriophage transduction of prokaryotic hosts. Alternatively, antibodies that bind to bacteriophage tail proteins and block their function can be used to demonstrate that wild type tail function is not required for the transfer of the bacteriophage genome to the host cell nucleus. The methods also are useful for identifying target cells that contain an appropriate receptor in sufficient quantity to internalize the preferred bacteriophage vectors via receptor-mediated endocytosis. The screening assays disclosed herein demonstrate that bacteriophage vectors which include one or more non-functional tail protein(s) and a standard genomic expression marker, e.g. beta-galactosidase or other detectable protein, are incapable of transferring genetic material to the target cells in the presence of the endocytosis inhibition agents but are capable of transduction when contacted with the target cells in the absence of such inhibition agents. Thus, the assay is useful for identifying novel bacteriophages which are structurally and functionally distinct from naturally-occurring bacteriophages and modified bacteriophage such as those described in PCT publication no. WO 96/21007, entitled, "Bacteriophage-mediated Gene Transfer Systems Capable of Transfecting Eukaryotic Cells", applicant Chiron Viagene, Inc. which report modified bacteriophages that inject their genetic contents into mammalian target cells.

Standard recombinant methods and screening technology are used to prepare a preferred bacteriophage vector with genetically modified tail protein genes that either contain amber mutations or sequences that enhance binding of the bacteriophage vector to the mammalian cell surface and subsequent internalization via receptor-mediated

endocytosis. Exemplary essential tail proteins in the lambda phage that can be modified to prepare preferred embodiments of the invention include: H, J, M proteins. As used herein, "essential tail proteins" refers to those proteins that are essential for facilitating the injection of the bacteriophage genetic material into its natural prokaryotic host. Although Applicants doubt the likelihood that bacteriophage can be selected or designed to have tail proteins that are capable of facilitating the injection of the bacteriophage genetic material into a eukaryotic host, the phrase "essential tail proteins" is also meant to embrace the tail proteins of such hypothetical eukaryotic cell-injecting bacteriophage. One or more of these or other tail proteins can be rendered non-functional (i.e., incapable of facilitating the injection of the genetic material into the host cell) using recombinant, mutagenesis, and/or chemical methods in accordance with procedures known to one of ordinary skill in the art. Such procedures can, of course, be applied to the preparation of other types of modified bacteriophages by, e.g., identifying the essential tail proteins of one or more bacteriophages in Table 1 and modifying the essential tail proteins as described herein.

The modifications to the essential tail protein(s) renders the bacteriophage incapable of mediating the transfer of genetic material into a mammalian host via an injection mechanism analogous to that responsible for the naturally occurring bacteriophage transduction of prokaryotic hosts. Preferably, such modification(s) of the tail proteins further render the bacteriophage incapable of infecting and/or propagating within its natural prokaryotic host (Su^0 E.Coli bacteria). Accordingly, such tail mutant bacteriophage vectors are packaged in vitro using specific purified protein packaging extracts and recombinant bacteriophage genomes or in Su^+ E. Coli bacteria (for amber mutant containing bacteriophage vectors). The benefit of such preferred bacteriophage vectors is that these vectors are incapable of propagating in the host organism's natural flora.

In certain embodiments, the tail proteins are modified so that they function to properly package the virus' genome but have lost their ability to mediate injection across bacterial cell membranes. Phages containing these modified tail-proteins require packaging in a cell-free system as described above.

In yet another embodiment, the mutations in the tail proteins are temperature sensitive such that at temperatures other than mammalian physiologic temperature, the tail

protein functions normally and can mediate prokaryotic cell infection; however, at mammalian physiologic temperature, the temperature sensitive mutation inactivates the wild type tail function. Such mutant bacteriophage vectors can be propagated in a prokaryotic host and packaged using standard procedures.

In a further embodiment, the tail protein is modified to include signals that target and/or facilitate entry of the vector's genetic material into subcellular organelles, including the nucleus. Exemplary signals for targeting the nucleus include polypeptides derived from the matrix or Vpr proteins of HIV or the large T-antigen of SV40. Such signals can be inserted into the lambda or other bacteriophage genomes using standard procedures. Preferably, such signals are integrated into the lambda phage genome in frame either within or appended to the J or H genes. For example, the genome of the modified bacteriophage described in Example 3 could be further modified in the following way. First using PCR fusion, a DNA fragment containing the SV40 nuclear localization sequence coding for NH₂-Pro-Lys-Lys-Lys-Arg-Lys- Val (PKKKRKV)(Kalderon D et al. Cell 1984;39: 499). (SEQ. ID. No. 7) flanked both 5' and 3' by wild type lambda DASH II J gene sequences including Bst 1170 I restriction sites is generated. Then this fusion DNA product is cut by Bst 1170 I leaving the following Bst 1170 I sticky end-5' J gene sequence-SV40/NLS-3' J gene sequence-Bst 1107 I sticky end. This digestion product is ligated, in frame, into the wild type Bst 1107 I restriction site within the J gene at base pair number 18834 by standard recombinant technology. Such signals can be inserted anywhere in the bacteriophage packaging protein coding sequence provided that such insertion does not interfere with receptor-mediated endocytosis and/or expression of the exogenous polynucleotide within the target cell. Optionally, insertion of these signals into the bacteriophage also inactivates wild type function and, optionally, further serves to enhance bacteriophage penetration of the outer membrane of the mammalian cell.

Example VI

Screening Method for Selecting Target Cells and Modified Bacteriophages of the Invention that Transduce Mammalian Target Cells via Receptor-mediated Endocytosis.

The following assay is useful for selecting target cells and bacteriophage vectors that depend on receptor mediated endocytosis for transfer of the bacteriophage genome to the target cell nucleus. The modified bacteriophages are grown to high titer 10^{12} to 10^{13} by standard methods employing either endogenous packaging in a prokaryotic host or in vitro packaging with proteins extracted from appropriately engineered lysogen strains. Additionally or alternatively, these modified bacteriophages may be modified chemically after packaging using standard methods. The coding sequence for beta-galactosidase or other easily detectable gene expression indicator(s) is incorporated into the genome of the test bacteriophages using standard procedures. At preferable multiplicities of infection of 1 to 100, purified bacteriophage is then added to sub-confluent cultures on cover slips of mammalian test cells that bear the receptor to which a modified bacteriophage has been targeted. One example of such a test cell line is HepG2 cells in which the asialoglycoprotein mediated endocytosis of galactose bearing oligosaccharides and macromolecules is well studied (described above).

The screening assay is performed in the presence and absence of endocytosis inhibitors. Preferably, prior to addition of the modified bacteriophage, inhibitors of endocytosis are added to the cell medium containing the target cells either singly or in various combinations at concentrations such that receptor mediated endocytosis is optimally inhibited without irreversibly injuring the test target cells. Exemplary endocytosis inhibitors include colchicine, taxol, monodansylcadaverine, cytochalasin B, or cytochalasin D. As a control, cells not treated with endocytosis inhibitors are infected with test bacteriophages in parallel.

The test cells are incubated with bacteriophage for about 48 hours or other suitable time to allow transduction and expression. At various time points during this period, the plated cells are fixed for x-gal staining, immunostaining, in situ hybridization, electron microscopy or other standard methods that are known to one skilled in the art for detecting the inserted exogenous polynucleotide or its expression product. X-gal staining is used to indicate whether or not the transduced genetic material (including a beta-galactosidase marker) is expressed in the test cells. Alternatively, immunostaining with specific antibodies or anti-sense in situ hybridization probes employed to detect either the translation or transcription of a gene inserted into the vector's genome. Additionally or

alternatively, antibodies with specific affinity for the modified bacteriophage surface protein(s) are used to detect the localization of viral coat proteins within test cells, including organelles within the test cells, using confocal immunofluorescence microscopy. Optionally, transmission electron microscopy is employed to determine the sub cellular localization of modified bacteriophages at various time points after introduction of the modified bacteriophage into the test cell media.

It is anticipated that less than about 5% of target cells that are contacted with the endocytosis inhibitor(s) demonstrate internalization and/or expression of the indicator gene. In addition, immunostaining with anti-coat antibodies of sections of test cells that have been exposed to endocytosis inhibitors and fixed directly following incubation with bacteriophage detect the presence of viral coat proteins in a sub-plasma membrane location, thus, indicating entrapment of these bacteriophages within endocytic vesicles prevented from fusion with lysosomes and normal trafficking. In contrast, the bacteriophage-infected test cells in which receptor-mediated endocytosis is not inhibited exhibit expression of the indicator gene in greater than about 15% of the test cells. Furthermore, immunostaining uninhibited test cell sections for coat proteins demonstrate a predominance of sub plasma membrane localization. These results are surprising and unexpected in view of the internalization mechanism proposed in PCT publication no. WO 96/21007 for bacteriophage transfer of genetic material to a mammalian cell.

Example VII:

Construction of a modified bacteriophage vector which displays cyclic RGD ligand on its surface

We have designed a bacteriophage genome which appends, in frame, the coding sequence cyclic RGD ligand 3' to the coding sequence of the native D-gene. This bacteriophage genome was packaged *in vitro* and propagated in *E. Coli* so as to produce productive phage which contains the D-gene-RGD fusion protein product on its surface. The effect of displaying this cyclic RGD ligand on the surface is to promote uptake of this bacteriophage by mammalian cells bearing the alpha-5/beta-1 integrin receptor via cell-mediated endocytosis. This modified bacteriophage is referred to as lambda DASH II-RGD. Using a beta-galactosidase reporting construct, we have found the lambda DASH II-RGD to infect 30-40% of cultured monolayer bovine endothelial cells at a multiplicity

of infection of 1000 phage particles per cell. The sequences of the RGD primers are shown in SEQ ID NOS. 18, 19, 20, and 21.

Example VIII:

Delivery and expression of human factor VIII/von Willebrand factor containing vector

As further enabling proof of our technology, we have constructed a bacteriophage genome which contains the following functional elements in 5' to 3' tandem array: CMV promoter-human factor VIII cDNA (Seq ID No. 15) - internal ribosomal entry site (IRES)-human von Willebrand factor cDNA (Seq. ID No. 17). The IRES cassette was obtained from Clontech (Palo Alto, CA). The total size of this linear DNA construct is 20.6 kilobases. As described in the detailed description of the invention, our FVIII/vWF construct has been spliced into the multiple cloning site of the bacteriophage lambda DASH II-RGD genome via blunt ended ligation (see included sequences and construct diagram).

This resulting genome has been efficiently packaged and propagated to titers of 10^{11} to 10^{12} phage particles per 2 liter broth culture. We have infected subconfluent monolayer cultures of bovine endothelial cells with this FVIII-vWF containing vector. Using a standard assay for factor VIII activity (COATEST VIII: C4-Chromogenenix Mölndal, Sweden), we have measured expression of factor VIII at 48 hours after infection. A 24 hour secretion study revealed that these transduced endothelial cells produce 56 mU/24 hours/10 cm culture plate (approximately 5×10^5 cells). This secretion rate is 8 fold greater than that reported for a transfected COS cell line (Toole et al., Nature, 312, 1984, p. 342-7).

Example IX: Delivery and expression of a murine dystrophin containing vector

As described in the examples of the patent application, we have obtained a 20.5 kilobase insert from Dr. Jeffrey Chamberlain (Michigan University) containing the following functional elements in tandem oriented 5' to 3': muscle creatine kinase promoter - murine full length dystrophin cDNA (Seq ID No. 17). We, in turn, have spliced this dystrophin insert into lambda DASH II-RGD via blunt end ligation into the multiple cloning site. The resulting recombinant bacteriophage genome has been packaged and

propagated efficiently to titers of 10^{11} to 10^{12} phage particles per 2 liter broth culture.

To test the expression capacity of this dystrophin bacteriophage vector, we injected 50 microliters of 10^{10} phage particles/mL into the gastrocnemius muscle of a dystrophin-deficient mouse (*mdx*). The phage vector was co-injected with a sublethal dose of India ink. This permitted accurate localization of the myocytes in proximity to the needle tract. Employing a polyclonal antibody against dystrophin (Cox et al. Nature, 264, 1993, 725-9), we have been able to demonstrate by immunofluorescence restoration of perimyocyte dystrophin expression. As previously reported (Ibid.), immunofluorescence study of the control uninjected *mdx* mouse muscle with the same anti-dystrophin antibody did not demonstrate specific perimyocyte immunoreactivity.

Each of the references, patents and patent publications disclosed in this document is incorporated in its entirety herein by reference.

While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is intended that such modification, changes and equivalents fall within the scope of the following claims.

The Tables are presented below and are followed by the Sequence Listing and what is claimed:

Table 1 Exemplary Bacteriophages

Lambda phage, p1 phage, T even and T odd phages (e.g., T1, T2, T3, T4, T5, T6 and T7); P2; P4; Mu; PM2; N4; SPO1; PBS1; PBS2; 29; SPP1; 6; PR4; PRD1; AP50; DS6A; I3; NS11; Dp-1; MVL2; CP-1; 434; cbk; G; D108; and P7.

Table 2

Exemplary Human Gene Therapy Protocols Approved by RAC

Disease	Gene Therapy Treatment	RAC OK
Severe combined immune deficiency (SCID) due to adenosine deaminase (ADA) deficiency	Autologous lymphocytes transduced with human ADA gene	7/31/90

Advanced cancer	Tumor-infiltrating lymphocytes transduced with tumor necrosis factor gene	7/31/90
Advanced cancer	Immunization with autologous cancer cells transduced with tumor necrosis factor gene	10/07/91
Advanced cancer	immunization with autologous cancer cells transduced with interleukin-2 gene	10/07/91
Familial hypercholesterolemia	<i>Ex vivo</i> gene therapy	10/08/91
Malignancy	<i>In vivo</i> gene transfer into tumors	2/10/92
Cancer	Gene transfer	2/10/92
Relapsed/refractory neuroblastoma	Cytokine-gene modified autologous neuroblastoma cells (Phase I study)	6/01/92
Brain tumors	Intratumoral transduction with thymidine kinase gene and intravenous ganciclovir	6/01/92
Metastatic melanoma	Immunization with HLA-A2 matched allogeneic melanoma cells that secrete interleukin-2	6/02/92
Advanced renal cell carcinoma	Immunization with interleukin-2 secreting allogeneic HLA-A2 matched renal-cell carcinoma cells	6/02/92
Cancer	Interleukin-4-gene modified antitumor vaccine (pilot study)	9/15/92
Cystic fibrosis	Replication deficient recombinant adenovirus carrying cDNA of normal human cystic fibrosis transmembrane conductance regulator (CFRT) gene; single administration to the lung (Phase I study)	12/03/92
Cystic fibrosis	El-deleted adenovirus vector for delivering CFTR gene (Phase I study)	12/03/92

Disease	Gene Therapy Treatment	RAC OK
Cystic fibrosis	Adenovirus vector used for delivering CFTR gene to nasal epithelium	12/04/92
Recurrent glioblastoma (brain tumor)	<i>In vivo</i> tumor transduction using herpes simplex thymidine kinase gene/ganciclovir system	3/01/93
Metastatic renal cell carcinoma	Injection of non-replicating autologous tumor cells prepared +/- granulocyte-macrophage colony stimulating factor transduction (Phase I study)	3/01/93
Cystic fibrosis	Use of replication deficient recombinant adenovirus vector to deliver human CFTR cDNA to the lungs (Phase I study)	3/02/93
Cystic fibrosis	Use of EI-deleted adenovirus for delivery of CFTR gene to nasal cavity (Phase I study)	3/02/93
Disseminated malignant melanoma	Human gamma-interferon transduced autologous tumor cells (Phase I study)	6/07/93
Ovarian cancer	Use of modified retro viruses to introduce chemotherapy resistance sequences into normal hematopoietic cells for chemoprotection (pilot study)	6/07/93
Cancer	Immunotherapy by direct gene transfer into tumors	6/07/93
Gaucher's disease	<i>Ex vivo</i> gene transfer and autologous transplantation of CD34 + cells	6/07/93
Gaucher's disease	Retro viral-mediated transfer of cDNA for human glucocerebrosidase into hematopoietic stem cells	6/07/93
Asymptomatic patients infected with HIV-1	Murine Retro viral vector encoding HIV-1 genes [HIV-IT(V)]	6/07/93

SUBSTITUTE SHEET (RULE 26)

AIDS	Effects of a transdominant form of <i>rev</i> gene on AIDS intervention	6/07/93
Recurrent pediatric malignant astrocytomas	<i>In vivo</i> tumor transduction with herpes simplex thymidine kinase gene	6/08/93
Advanced cancer	Human multiple-drug resistance (MDR) gene transfer	6/08/93
Brain tumors	Episome-based antisense cDNA transcription of insulin-like growth factor I	6/08/93
Small-cell lung cancer	Cancer cells transfected with and expressing interleukin-2 gene (Phase I study)	9/09/93

-55/1-

Disease	Gene Therapy Treatment	RAC OK
Breast cancer (post-chemotherapy)	Retro viral mediated transfer of the human MDR gene into hematopoietic stem cells (autologous transplantation)	9/09/93
Recurrent pediatric brain tumors	Intra-tumoral transduction with thymidine kinase gene and intravenous administration of ganciclovir	9/09/93
Malignant melanoma	Immunization with interleukin-2 secreting allogeneic human melanoma cells	9/10/93
HIV infection	Autologous lymphocytes transduced with catalytic ribozyme that cleaves HIV-1 RNA (Phase I study)	9/10/93
Metastatic melanoma	Genetically engineered autologous tumor vaccines producing interleukin-2	9/10/93
Leptomeningeal carcinomatosis	Intrathecal gene therapy	12/02/93
Colon carcinoma	Injection with autologous irradiated tumor cells and fibroblasts genetically modified to secrete interleukin-2	12/2/93
Gaucher's disease	Retro virus-mediated transfer of cDNA for human glucocerebrosidase into peripheral blood repopulating patients' cells	12/3/93
HIV infection	Murine Retro viral vector encoding HIV-IT(V) genes (open label Phase I/II trial)	12/03/93
Advanced (stage IV) melanoma	Induction of cell-mediated immunity against tumor-associated antigens by B7-transfected lethally irradiated allogeneic melanoma cell lines (Phase I study)	12/03/93

SUBSTITUTE SHEET (RULE 26)

-55/2-

Advanced colorectal carcinoma	Immunotherapy by direct gene transfer into hepatic metastases (Phase I study)	12/03/93
Melanoma	Adoptive immunotherapy with activated lymph node cells primed <i>in vivo</i> with autologous tumor cells transduced with interleukin-4 gene	12/03/93
Cystic fibrosis	Cationic liposome-mediated transfer of CFTR gene into nasal airway (Phase I study)	12/03/93
Cystic fibrosis	Adenovirus-mediated transfer of CFTR gene to the nasal epithelium and maxillary sinus	12/03/93
Pediatric neuroblastoma	Immunization with gamma-interferon transduced neuroblastoma cells (<i>ex vivo</i>) (Phase I)	3/03/94

SUBSTITUTE SHEET (RULE 26)

Table 3

Preferred Therapeutic Polynucleotides and Corresponding Medical Conditions

Dystrophin-Duchenne muscular dystrophy
5 Globin gene complex-Hemoglobinopathies, e.g. sickle cell anemia, thalassemias
Clotting factor VIII-Hemophilia A
von Willebrand's factor-von Willebrand's disease
Collagen type VII-Epidermolysis bullosa dystrophica
Combinations of cyclin dependent kinase inhibitors, and other cell cycle inhibitors,
10 e.g. p15, p16, p18-Neoplastic processes, e.g. melanoma
Fibrillin-Marfan's syndrome
Polypeptide antigens-Vaccines for tumors, infectious agents
Combinations of cytokines or co-stimulatory immune modulators, e.g., IL-1, IL-2, IL12,
GM-CSF, TNF α , IL4, B7-Neoplastic processes
15 Thymidine kinase-Suicide gene for neoplastic, hyperplastic or hypertrophic processes
Combinations of ribozymes-Targeted against disease predisposing MHC genes or against
disease associated messenger RNAs of viral origin, e.g. E6, E7 oncoproteins
in HPV, reverse transcriptase in HIV
Individual or combinations of chemotherapy resistance genes to protect bone marrow
20 stem cells from chemotherapy regimens
Polypeptide antigens in conjunction with tolerance inducing sequences, e.g. ribozyme
against B7-1-Treatment for autoimmune disease, e.g. rheumatoid arthritis,
psoriasis, multiple sclerosis, alopecia areata
Combinations of ribozyme(s), antisense RNA(s), or polypeptide coding sequences(s) for
25 biopolymers that interfere with human viral infections, e.g. HIV, CMV, Hepatitis
B and C, Herpes Simplex I, II, HHV-8, EBV, HTLV-I
Combinations of ribozyme(s), antisense RNA(s), or polypeptide coding sequences(s) for
biopolymers that interfere with chronic bacterial or parasitic infections, e.g.
leprosy, tuberculosis, antibiotic resistant bacteria (e.g., MRSA, VRE),
30 trypanosomiasis, filaraisis, and the like

Table 4

Exemplary Target Cells

Hepatocytes
5 Melanocytes
Keratinocytes
Myocytes
Adipocytes
Hematopoietic cells, e.g. lymphocytes, erythrocytes, leukocytes, monocytes, progenitor
10 and stem cells
Neurons
Glial cells
Antigen presenting cells, e.g. macrophages, B-cells, Langerhan's cells
Chondrocytes
15 Osteocytes
Osteoclasts
Endothelial cells
Phagocytes
Fibroblasts
20 Smooth muscle cells
Renal tubule cells
Mesangial cells
Thymocytes
Bronchopulmonary, gastrointestinal, breast, genitourinary, corneal, renal ductular
25 epithelial cells
Endocrine, exocrine gland cells
Plasma cells
Mast cells
Lens epithelial cells
30 Retinal epithelial cells
Malignant cells of any derivation
Placental cells
Gonadal cells
Embryonic cells
35 Zygotes

Table 5

Exemplary Promoters and EnhancersConstitutive

Phosphoglycerokinase

Long terminal repeat (LTR) of retroviruses, e.g. Moloney murine leukemia virus,
Rous sarcoma virus

Cytomegalovirus promoter

Hematopoietic cells

Promoters

c fms (monocytes, trophoblasts)

T-cell receptor

Enhancers

Immunoglobulin heavy chain

Locus control region of the globin gene complex CD2

Hepatocytes

Promoters

Albumin

 α -1-antitrypsin

Pyruvate kinase

Phosphoenol pyruvate carboxykinase

Transferrin

Transthyretin

 α -fetoprotein α -fibrinogen β -fibrinogen

Hepatitis B

Enhancers

Hepatitis B

Tyrosine aminotransferase

Cardiac myocytes

Promoter

Myosin light chain-2

 β -myosin heavy chain (cardiac and slow twitch skeletal) α -cardiac myosin heavy chain

Cardiac alpha actin

Enhancer

 β -myosin heavy chainFibroblasts

Promoter

Collagen alpha-2 (I)

Elastin (fibroblasts and smooth muscle cells)

Neurons

Promoter

Peripheral myelin protein-22

Adipocytes

Promoter

Lipoprotein lipase

Aromatase cytochrome P450 (adipocytes, brain, ovary)

Thyroid

Promoter

Thyroglobulin

Lens epithelium

Promoter

Crystallin

Breast epithelium

Promoter

Milk protein gene

Skeletal muscle

Promoter

Glut-4

Muscle creatine kinase (skeletal and cardiac muscle)

Enhancer

Muscle creatine kinase (skeletal and cardiac muscle)

Urinary bladder

Promoter

UroplakinII

Keratinocyte

Promoter

Keratin 14

Keratin 10

Involucrin

Melanocyte

Promoter

Tyrosinase

Non specific enhancer elements

SV40

CMV

LTR

Inducible or repressible promoter systems

Estrogen-Gal4 inducible system

RU486-Gal4 inducible system

Tetracycline inducible system

IPTG system

Metallothionein

Tetracycline repressible system

Table 6
Exemplary Receptors and Preferred Ligands

Hepatic receptors

5 hyaluronic acid
collagen
N-terminal propeptide of collagen type III
mannose/N-acetylglucosamine
complement
10 asialoglycoprotein
tissue plasminogen activator
low density lipoprotein
insulin
ceruloplasmin
15 enterokinase
carcinoembryonic antigen
apamin
galactose/lactose

20 Growth Factor/Cytokine receptors

hepatocyte growth factor
epidermal growth factor
insulin-like growth factor I, II
interleukin-1 a/b
25 interleukin-2, IL-7, IL-4
 γ -interferon
 β -interferon
keratinocyte growth factor
TNF-R p55

30 Hormone receptors

prolactin
thyroglobulin
growth hormone
35 insulin
glucagon
leutinizing hormone
human choriogonadotrophic hormone

40 Nerve cell receptors

neurotensin

Antigen presenting cell receptors

45 immunoglobulin G-Fc receptor

Kidney cells

angiotensin II

vasopressin

Bone marrow receptors

c kit

CD-34

Keratinocyte and skin fibroblast receptors

very low density lipoprotein

low density lipoprotein

integrins that bind to RGD bearing polypeptides

collagen

laminin

Placental receptors

hemopexin

immunoglobulin G-Fc

low density lipoprotein

transferrin

alpha2-macroglobulin

ferritin

insulin

γ-interferon

epidermal growth factor

insulin-like growth factor

Muscle cell receptors

insulin

very low density lipoprotein

Gut epithelium

cobalamin-intrinsic factor

heat stable enterotoxin of E. Coli

Breast epithelium

heregulin

prolactin

Melanocytes

c kit

Miscellaneous

folate

cobalamin (B12)

Preferred ligands

low density lipoprotein (apoprotein B100)
very low density lipoprotein (apoprotein E)
galactose
c kit ligand
transferrin
insulin
heregulin
RGD or RGD-containing polypeptides

Table 7Exemplary Antigens to Induce or Enhance an Immune Response

Melanoma or other tumor specific antigens;
leishmaniasis antigens;
helicobacter pylori specific antigens (e.g., urease B);
hepatitis B antigens;
hepatitis C antigens;
Herpes simplex antigens;
HIV antigens;
Tuberculosis antigens;
cytomegalovirus antigens;
lyme disease antigens;
malaria antigens;
respiratory syncytial virus antigens;
leprosy antigens;
toxoplasmosis antigens;
pneumocystis carinii antigens;
schistosomiasis antigens;
chlamydial antigens;
HTLV-1 antigens;
enterococcal antigens (e.g., VRE);
gonococcal antigens;
treponemal antigens;
clostridium difficile antigens;
Staphylococcus aureus antigens (e.g., MRSA);
trypanosomal antigens;
filarial antigens;
salmonella antigens;
shigella antigens;
pneumococcal antigens (e.g., penicillin resistant strains);
pseudomonal antigens

Table 8

Advantages of the Targeted Phage Vectors Over
the Vectors Currently Used in Gene Therapy

	Size Constraints	Specificity of Targeting	Immunogenicity/ Toxicity	Sustained/High/Low/ Controlled Expression
Retrovirus Vector	7Kb	none	none	low, uncontrolled transient transfection
Adenovirus Vector	7Kb	none	high immunogenicity	low, uncontrolled transient transfection
Liposome	none	none	toxic at high doses	low, uncontrolled transient transfection
Phage Vectors	bacteriophage lambda DASH II (up to 23Kb); Minimal lambda cassette (up to 50 kb); P1 (up to 95 Kb)	yes	none	controlled high persistent expression

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: BRIGHAM AND WOMEN'S HOSPITAL, INC.
(B) STREET: 75 FRANCIS STREET
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE: 02115

(i) APPLICANT/INVENTOR:

- (A) NAME: SARKAR, SAUMYENDRA N.
(B) STREET: 102 QUEENSBURY STREET
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE: 02115

(i) APPLICANT/INVENTOR:

- (A) NAME: KUPPER, THOMAS S.
(B) STREET: 8 SURREY LANE
(C) CITY: WESTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE: 02193

(i) APPLICANT/INVENTOR:

- (A) NAME: DUBIN, DANIEL B.
(B) STREET: 99 POND AVE
(C) CITY: BROOKLINE
(D) STATE: MASSACHUSETTS
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE: 02146

(ii) TITLE OF THE INVENTION: BACTERIOPHAGE-MEDIATED GENE
THERAPY

(iii) NUMBER OF SEQUENCES: 21

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: WOLF, GREENFIELD & SACKS, P.C.
(B) STREET: 600 ATLANTIC AVENUE
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS

-66-

(E) COUNTRY: UNITED STATES OF AMERICA

(F) POSTAL CODE: 02210

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/693,865

(B) FILING DATE: 08-MAY-1996

(A) APPLICATION NUMBER: US 08/814,859

(B) FILING DATE: 11-MAR-1997

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Plumer, Elizabeth R.

(B) REGISTRATION NUMBER: 36,637

(C) REFERENCE/DOCKET NUMBER: B0801/7059WO

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-720-3500

(B) TELEFAX: 617-720-2441

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacteriophage lambda

SUBSTITUTE SHEET (RULE 26)

-67-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATACCGAGGG CTGCAGTGTA CA

22

5 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

20 (A) ORGANISM: Bacteriophage lambda

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTCTTTCAAT TGGGGAGGCA AAACGATGCT GATTGCCGTT C

41

25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

30 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

40

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

45 TTGCCTCCCC AATTGAAAGA G

21

(2) INFORMATION FOR SEQ ID NO:4:

SUBSTITUTE SHEET (RULE 26)

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 60 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGATGAAGG GTAAAGTTAT TTGCGTTTTT TTTTCGGCGG GGTCTCCAT AAATTCATC

60

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Bacteriophage lambda

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAACTTTACC CTTTCATCACT AAAGGCC

27

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

SUBSTITUTE SHEET (RULE 26)

-69-

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacteriophage lambda

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAACGTACAG CGCCATGTTT ACCAG

25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Pro Lys Lys Lys Arg Lys Val

1

5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Ser Arg Lys Arg Pro Arg Pro

1

5

(2) INFORMATION FOR SEQ ID NO:9:

SUBSTITUTE SHEET (RULE 26)

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Arg Pro Ala Ala Thr Lys Lys Ala Gly Gln Ala Lys Lys Lys Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Arg Thr Thr Lys Gly Lys Arg Lys Arg Ile Asp Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

SUBSTITUTE SHEET (RULE 26)

Ala Ala Lys Arg Val Lys Leu Asp
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Ser Ser Lys Arg Pro Arg Pro
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Lys Ile Arg Leu Arg Pro Gly Gly Lys Lys Lys Tyr Lys Leu Lys His
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 300 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

5	Met	Thr	Ser	Arg	Arg	Ser	Val	Lys	Ser	Gly	Pro	His	Glu	Val	Pro	Arg	1	5	10	15
	Gln	Glu	Tyr	Glu	Asp	Leu	Tyr	Tyr	Thr	Pro	Ser	Ser	Gly	Met	Ala	Ser	20	25	30	
10	Pro	Gln	Ser	Pro	Pro	Gln	Thr	Ser	Arg	Arg	Gly	Ala	Leu	Gln	Thr	Arg	35	40	45	
	Ser	Arg	Gln	Arg	Gly	Glu	Val	Arg	Phe	Val	Gly	Tyr	Asp	Glu	Ser	Asp	50	55	60	
15	Tyr	Ala	Leu	Tyr	Gly	Gly	Ser	Ser	Ser	Glu	Asp	Asp	Glu	His	Pro	Glu	65	70	75	80
	Pro	Pro	Thr	Arg	Arg	Pro	Val	Ser	Gly	Ala	Val	Ala	Ser	Gly	Pro	Gly	85	90	95	
20	Pro	Ala	His	Ala	Pro	Pro	Pro	Pro	Ala	Gly	Ser	Gly	Gly	Ala	Gly	Arg	100	105	110	
	Thr	Pro	Thr	Thr	Ala	Pro	Arg	Ala	Pro	Arg	Thr	Gln	Arg	Val	Ala	Thr	115	120	125	
25	Lys	Ala	Pro	Ala	Ala	Pro	Ala	Ala	Glu	Thr	Thr	Arg	Gly	Arg	Lys	Ser	130	135	140	
30	Ala	Gln	Pro	Glu	Ser	Ala	Ala	Leu	Pro	Gln	Ala	Pro	Ala	Ser	Thr	Ala	145	150	155	160
	Arg	Thr	Arg	Ser	Lys	Thr	Pro	Ala	Gly	Gly	Leu	Ala	Arg	Lys	Leu	His	165	170	175	
35	Glu	Ser	Thr	Ala	Pro	Pro	Asn	Pro	Asp	Ala	Pro	Val	Val	Thr	Pro	Arg	180	185	190	
40	Val	Ala	Gly	Phe	Asn	Lys	Arg	Val	Cys	Ala	Ala	Val	Gly	Arg	Leu	Ala	195	200	205	
	Ala	Met	His	Ala	Arg	Met	Ala	Ala	Val	Gln	Leu	Val	Val	Asp	Met	Ser	210	215	220	
45	Arg	Pro	Arg	Ile	Asp	Glu	Asp	Ile	Asn	Glu	Leu	Leu	Gly	Ile	Thr	Thr	225	230	235	240

SUBSTITUTE SHEET (RULE 26)

Ile Arg Val Thr Val Cys Glu Gly Lys Asn Leu Leu Gln Arg Ala Asn
245 250 255

Glu Val Asn Pro Asp Val Val Gln Asp Val Asp Ala Ala Thr Ala Thr
260 265 270

Arg Gly Arg Ser Ala Ala Ser Arg Pro Thr Glu Arg Pro Arg Ala Pro
275 280 285

Ala Pro Ser Ala Ser Arg Pro Arg Arg Pro Val Glu
290 295 300

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9009 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAGTGGGTAA	GTTCCTTAAA	TGCTCTGCAA	AGAAATTGGG	ACTTTTTCATT	AAATCAGAAA	60
TTTTACTTTT	TTCCCCTCCT	GGGAGCTAAA	GATATTTTAG	AGAAGAATTA	ACCTTTTGCT	120
TCTCCAGTTG	AACATTTGTA	GCAATAAGTC	ATGCAAATAG	AGCTCTCCAC	CTGCTTCTTT	180
CTGTGCCCTT	TGCGATTCTG	CTTTAGTGCC	ACCAGAAGAT	ACTACCTGGG	TGCAGTGGAA	240
CTGTTCATGGG	ACTATATGCA	AAGTGATCTC	GGTGAGCTGC	CTGTGGACGC	AAGATTTCCT	300
CCTAGAGTGC	CAAAATCTTT	TCCATTCAAC	ACCTCAGTCG	TGTACAAAAA	GACTCTGTTT	360
GTAGAATTCA	CGGTTACCT	TTTCAACATC	GCTAAGCCAA	GGCCACCCTG	GATGGGTCTG	420
CTAGGTCCTA	CCATCCAGGC	TGAGGTTTAT	GATACAGTGG	TCATTACACT	TAAGAACATG	480
GCTTCCCATC	CTGTCAGTCT	TCATGCTGTT	GGTGTATCCT	ACTGGAAAGC	TTCTGAGGGA	540
GCTGAATATG	ATGATCAGAC	CAGTCAAAGG	GAGAAAGAAG	ATGATAAAGT	CTTCCCTGGT	600
GGAAGCCATA	CATATGTCTG	GCAGGTCCTG	AAAGAGAATG	GTCCAATGGC	CTCTGACCCA	660
CTGTGCCTTA	CCTACTCATA	TCITTTCTCAT	GTGGACCTGG	TAAAAGACTT	GAATTCAGGC	720
CTCATTGGAG	CCCTACTAGT	ATGTAGAGAA	GGGAGTCTGG	CCAAGGAAAA	GACACAGACC	780
TTGCACAAAT	TTATACTACT	TTTGTCTGTA	TTTGATGAAG	GGAAAAGTTG	GCACTCAGAA	840
ACAAAGAACT	CCTTGATGCA	GGATAGGGAT	GCTGCATCTG	CTCGGGCCTG	GCCTAAAATG	900
CACACAGTCA	ATGGTTATGT	AAACAGGTCT	CTGCCAGGTC	TGATTGGATG	CCACAGGAAA	960
TCAGTCTATT	GGCATGTGAT	TGGAATGGGC	ACCACTCCTG	AAGTGCCTC	AATATTCCCTC	1020
GAAGGTCACA	CATTTCTTGT	GAGGAACCAT	CGCCAGGCGT	CCTTGGAAT	CTCGCCAATA	1080
ACTTTTCCTTA	CTGCTCAAAC	ACTCTTGATG	GACCTTGGAC	AGTTTCTACT	GTTTTGTTCAT	1140
ATCTCTTCCC	ACCAACATGA	TGGCATGGAA	GCITATGTCA	AAGTAGACAG	CTGTCCAGAG	1200
GAACCCCAAC	TACGAATGAA	AAATAATGAA	GAAGCGGAAG	ACTATGATGA	TGATCTTACT	1260

SUBSTITUTE SHEET (RULE 26)

	GATTCTGAAA	TGGATGTGGT	CAGGTTTGAT	GATGACAACT	CTCCTTCCTT	TATCCAAATT	1320
	CGCTCAGTTG	CCAAGAAGCA	TCCTAAAAC	TGGGTACATT	ACATTGCTGC	TGAAGAGGAG	1380
	GACTGGGACT	ATGCTCCCTT	AGTCCTCGCC	CCCGATGACA	GAAGTTATAA	AAGTCAATAT	1440
	TTGAACAATG	GCCCTCAGCG	GATTGGTAGG	AAGTACAAAA	AAGTCCGATT	TATGGCATAC	1500
5	ACAGATGAAA	CCTTTAAGAC	TCGTGAAGCT	ATTGAGCATG	AATCAGGAAT	CTTGGGACCT	1560
	TTACTTTTATG	GGGAAGTTGG	AGACACACTG	TTGATTATAT	TTAAGAATCA	AGCAAGCAGA	1620
	CCATATAACA	TCTACCCTCA	CGGAATCACT	GATGTCCGTC	CTTTGTATTTC	AAGGAGATTA	1680
	CCAAAAGGTG	TAAAACATTT	GAAGGATTTT	CCAATTCTGC	CAGGAGAAAT	ATTCAAATAT	1740
	AAATGGACAG	TGACTGTAGA	AGATGGGCCA	ACTAAATCAG	ATCCTCGGTG	CCTGACCCGC	1800
10	TATTACTCTA	GTTTCGTTAA	TATGGAGAGA	GATCTAGCTT	CAGGACTCAT	TGGCCCTCTC	1860
	CTCATCTGCT	ACAAAGAATC	TGTAGATCAA	AGAGGAAACC	AGATAATGTC	AGACAAGAGG	1920
	AATGTCATCC	TGTTTTCTGT	ATTTGATGAG	AACCGAAGCT	GGTACCTCAC	AGAGAATATA	1980
	CAACGCTTTC	TCCCCAATCC	AGCTGGAGTG	CAGCTTGAGG	ATCCAGAGTT	CCAAGCCTCC	2040
	AACATCATGC	ACAGCATCAA	TGGCTATGTT	TTTGATAGTT	TGCAGTTGTC	AGTTTGTTTG	2100
15	CATGAGGTGG	CATACTGGTA	CAITCTAAGC	ATTGGAGCAC	AGACTGACTT	CCTTCTGTCT	2160
	TTCTTCTCTG	GATATACCTT	CAAACACAAA	ATGGTCTATG	AAGACACACT	CACCCTATTTC	2220
	CCATTCTCAG	GAGAACTGT	CTTCATGTCT	ATGGAAAACC	CAGGTCTATG	GATTCTGGGG	2280
	TGCCACAAC	CAGACTTTCG	GAACAGAGGC	ATGACCGCCT	TACTGAAGGT	TTCTAGTTGT	2340
	GACAAGAACA	CTGGTGATTA	TTACGAGGAC	AGTTATGAAG	ATATTTTCAGC	ATACTTGCTG	2400
20	AGTAAAAACA	ATGCCATTGA	ACCAAGAAGC	TTCTCCCAGA	ATTCAAGACA	CCCTAGCACT	2460
	AGGCAAAAGC	AATTTAATGC	CACCACAATT	CCAGAAAATG	ACATAGAGAA	GACTGACCCT	2520
	TGGTTTGCAC	ACAGAACACC	TATGCCTAAA	ATACAAAATG	TCTCCTCTAG	TGATTTGTTG	2580
	ATGCTCTTGC	GACAGAGTCC	TACTCCACAT	GGGCTATCCT	TATCTGATCT	CCAAGAAGCC	2640
	AAATATGAGA	CTTTTTCTGA	TGATCCATCA	CCTGGAGCAA	TAGACAGTAA	TAACAGCCTG	2700
25	TCTGAAATGA	CACACTTCAG	GCCACAGCTC	CATCACAGTG	GGGACATGGT	ATTTACCCCT	2760
	GAGTCAGGCC	TCCAATTAA	ATTAAATGAG	AAACTGGGGA	CAACTGCAGC	AACAGAGTTG	2820
	AAGAACTTG	ATTTCAAAGT	TTCTAGTACA	TCAAATAATC	TGATTTCAAC	AATTCCATCA	2880
	GACAATTTGG	CAGCAGGTAC	TGATAATACA	AGTTCCCTAG	GACCCCCAAG	TATGCCAGTT	2940
	CATTATGATA	GTCAATTAGA	TACCACTCTA	TTTGGCAAAA	AGTCATCTCC	CCTTACTGAG	3000
30	TCTGGTGGAC	CTCTGAGCTT	GAGTGAAGAA	AATAATGATT	CAAAGTTGTT	AGAATCAGGT	3060
	TTAATGAATA	GCCAAGAAAG	TTCATGGGGA	AAAAATGTAT	CGTCAACAGA	GAGTGGTAGG	3120
	TTATTTAAAG	GGAAAAGAGC	TCATGGACCT	GCTTTGTTGA	CTAAAGATAA	TGCCTTATTTC	3180
	AAAGTTAGCA	TCTCTTTGTT	AAAGACAAAC	AAAACCTCCA	ATAATTCAGC	AACTAATAGA	3240
	AAGACTCACA	TTGATGGCCC	ATCATTATTA	ATTGAGAATA	GTCCATCAGT	CTGGCAAAAT	3300
35	ATATTAGAAA	GTGACACTGA	GTTTAAAAAA	GTGACACCTT	TGATTTCATGA	CAGAATGCTT	3360
	ATGGACAAAA	ATGCTACAGC	TTTGAGGCTA	AATCATATGT	CAAATAAAAC	TACTTCATCA	3420
	AAAAACATGG	AAATGGTCCA	ACAGAAAAAA	GAGGGCCCCA	TTCCACCAGA	TGCACAAAAT	3480
	CCAGATATGT	CGTTCTTTAA	GATGCTATTTC	TTGCCAGAAT	CAGCAAGGTG	GATACAAAGG	3540
	ACTCATGGAA	AGAACTCTCT	GAACTCTGGG	CAAGGCCCCA	GTCCAAAGCA	ATTAGTATCC	3600
40	TTAGGACCAG	AAAAATCTGT	GGAAGGTCAG	AATTTCTTGT	CTGAGAAAAA	CAAAGTGGTA	3660
	GTAGGAAAGG	GTGAATTTAC	AAAGGACGTA	GGACTCAAAG	AGATGGTTTT	TCCAAGCAGC	3720
	AGAAACCTAT	TTCTTACTAA	CTTGGATAAT	TTACATGAAA	ATAATACACA	CAATCAAGAA	3780
	AAAAAAATTC	AGGAAGAAAT	AGAAAAGAAG	GAAACATTAA	TCCAAGAGAA	TGTAGTTTTG	3840
	CCTCAGATAC	ATACAGTGAC	TGGCACTAAG	AATTTTCATGA	AGAACCTTTT	CTTACTGAGC	3900
45	ACTAGGCAAA	ATGTAGAAGG	TTCATATGAG	GGGGCATATG	CTCCAGTACT	TCAAGATTTT	3960
	AGGTCATTAA	ATGATTCAAC	AAATAGAACA	AAGAAACACA	CAGCTCATTT	CTCAAAAAAA	4020
	GGGGAGGAAG	AAAACCTGGA	AGGCTTGGGA	AATCAAACCA	AGCAAAATGT	AGAGAAATAT	4080

SUBSTITUTE SHEET (RULE 26)

	GCATGCACCA	CAAGGATATC	TCCTAATACA	AGCCAGCAGA	ATTTTGTCAC	GCAACGTAGT	4140
	AAGAGAGCTT	TGAAACAATT	CAGACTCCCA	CTAGAAGAAA	CAGAACTTGA	AAAAAGGATA	4200
	ATTGTGGATG	ACACCTCAAC	CCAGTGGTCC	AAAAACATGA	AACATTTGAC	CCCGAGCACC	4260
	CTCACACAGA	TAGACTACAA	TGAGAAGGAG	AAAGGGGCCA	TTACTCAGTC	TCCCTTATCA	4320
5	GATTGCCTTA	CGAGGAGTCA	TAGCATCCCT	CAAGCAAATA	GATCTCCATT	ACCCATTGCA	4380
	AAGGTATCAT	CATTTCCATC	TATTAGACCT	ATATATCTGA	CCAGGGTCCT	ATTCCAAGAC	4440
	AACTCTTCTC	ATCTTCCAGC	AGCATCTTAT	AGAAAGAAAG	ATTCTGGGGT	CCAAGAAAGC	4500
	AGTCATTTCT	TACAAGGAGC	CAAAAAAAT	AACCTTTCTT	TAGCCATTCT	AACCTTGGAG	4560
	ATGACTGGTG	ATCAAAGAGA	GGTTGGCTCC	CTGGGGACAA	GTGCCACAAA	TTCAAGTCACA	4620
10	TACAAGAAAG	TTGAGAACAC	TGTTCTCCCG	AAACCAGACT	TGCCCAAAAC	ATCTGGCAAA	4680
	GTTGAATTGC	TTCCAAAAGT	TCACATTTAT	CAGAAGGACC	TATTCCCTAC	GGAAACTAGC	4740
	AATGGGTCTC	CTGGCCATCT	GGATCTCGTG	GAAGGGAGCC	TTCTTCAGGG	AACAGAGGGA	4800
	GCGATTAAGT	GGAATGAAGC	AAACAGACCT	GGAAAAGTTC	CCTTTCTGAG	AGTAGCAACA	4860
	GAAAGCTCTG	CAAAGACTCC	CTCCAAGCTA	TTGGATCCTC	TTGCTTGGGA	TAACCACTAT	4920
15	GGTACTCAGA	TACCAAAAGA	AGAGTGGAAA	TCCAAGAGA	AGTCACCAGA	AAAAACAGCT	4980
	TTTAAGAAAA	AGGATACCAT	TTTGTCCCTG	AACGCTTG TG	AAAGCAATCA	TGCAATAGCA	5040
	GCAATAAATG	AGGGACAAAA	TAAGCCCGAA	ATAGAAGTCA	CCTGGGCAAA	GCAAGGTAGG	5100
	ACTGAAAGGC	TGTGCTCTCA	AAACCCACCA	GTCTTGAAAC	GCCATCAACG	GGAAATAACT	5160
	CGTACTACTC	TTCAGTCAGA	TCAAGAGGAA	ATTGACTATG	ATGATACCAT	ATCAGTTGAA	5220
20	ATGAAGAAGG	AAGATTTTGA	CATTTATGAT	GAGGATGAAA	ATCAGAGCCC	CCGCAGCTTT	5280
	CAAAAGAAAA	CACGACACTA	TTTTATTGCT	GCAGTGGAGA	GGCTCTGGGA	TTATGGGATG	5340
	AGTAGCTCCC	CACATGTTCT	AAGAAACAGG	GCTCAGAGTG	GCAGTGTCCC	TCAGTTCAAG	5400
	AAAGTTGTTT	TCCAGGAATT	TACTGATGGC	TCCTTTACTC	AGCCCTTATA	CCGTGGAGAA	5460
	CTAAATGAAC	ATTTGGGACT	CCTGGGGCCA	TATATAAGAG	CAGAAGTTGA	AGATAATATC	5520
25	ATGGTAACCT	TCAGAAATCA	GGCCTCTCGT	CCCTATTCTT	TCTATTCTAG	CCTTATTTCT	5580
	TATGAGGAAG	ATCAGAGGCA	AGGAGCAGAA	CCTAGAAAAA	ACTTTGTCAA	GCCTAATGAA	5640
	ACCAAAACTT	ACTTTTGGAA	AGTGCAACAT	CATATGGCAC	CCACTAAAGA	TGAGTTTGAC	5700
	TGCAAAGCCT	GGGCTTATTT	CTCTGATGTT	GACCTGGAAA	AAGATGTGCA	CTCAGGCCTG	5760
	ATTGGACCCC	TTCTGGTCTG	CCACACTAAC	ACACTGAACC	CTGCTCATGG	GAGACAAGTG	5820
30	ACAGTACAGG	AATTTGCTCT	GTTTTTCACC	ATCTTTGATG	AGACCAAAAG	CTGGTACTTC	5880
	ACTGAAAATA	TGGAAAGAAA	CTGCAGGGCT	CCCTGCAATA	TCCAGATGGA	AGATCCCACT	5940
	TTTAAAGAGA	ATTATCGCTT	CCATGCAATC	AATGGCTACA	TAATGGATAC	ACTACCTGGC	6000
	TTAGTAATGG	CTCAGGATCA	AAGGATTCTG	TGGTATCTGC	TCAGCATGGG	CAGCAATGAA	6060
	AACATCCATT	CTATTCAATT	CAGTGGACAT	GTGTTCACTG	TACGAAAAAA	AGAGGAGTAT	6120
35	AAAATGGCAC	TGTACAATCT	CTATCCAGGT	GTTTTTGAGA	CAGTGGAAAT	GTTACCATCC	6180
	AAAGCTGGAA	TTTGGCGGGT	GGAATGCCTT	ATTGGCGAGC	ATCTACATGC	TGGGATGAGC	6240
	ACACTTTTTT	TGGTGTACAG	CAATAAGTGT	CAGACTCCCC	TGGGAATGGC	TTCTGGACAC	6300
	ATTAGAGATT	TTTCAATTAC	AGCTTCAGGA	CAATATGGAC	AGTGGGCCCC	AAAGCTGGCC	6360
	AGACTTCATT	ATTCCGGATC	AATCAATGCC	TGGAGCACCA	AGGAGCCCTT	TTCTTGGATC	6420
40	AAGGTGGATC	TGTTGGCACC	AATGATTATT	CACGGCATCA	AGACCCAGGG	TGCCCGTCAG	6480
	AAGTTCTCCA	GCCTCTACAT	CTCTCAGTTT	ATCATCATGT	ATAGTCTTGA	TGGGAAGAAG	6540
	TGGCAGACTT	ATCGAGGAAA	TTCCACTGGA	ACCTTAATGG	TCTTCTTTGG	CAATGTGGAT	6600
	TCATCTGGGA	TAAAACACAA	TATTTTAAAC	CCTCCAATTA	TTGCTCGATA	CATCCGTTTG	6660
	CACCCAACTC	ATTATAGCAT	TCGCAGCACT	CTTCGCATGG	AGTTGATGGG	CTGTGATTTA	6720
45	AATAGTTGCA	GCATGCCATT	GGGAATGGAG	AGTAAAGCAA	TATCAGATGC	ACAGATTACT	6780
	GCTTCATCCT	ACTTTTACCA	TATGTTTGCC	ACCTGGTCTC	CTTCAAAGC	TCGACTTCAC	6840
	CTCCAAGGGA	GGAGTAATGC	CTGGAGACCT	CAGGTGAATA	ATCCAAAAGA	GTGGCTGCAA	6900

SUBSTITUTE SHEET (RULE 26)

	GTGGACTTCC	AGAAGACAAT	GAAAGTCACA	GGAGTAACTA	CTCAGGGAGT	AAAATCTCTG	6960
	CTTACCAGCA	TGTATGTGAA	GGAGTTCCTC	ATCTCCAGCA	GTCAAGATGG	CCATCAGTGG	7020
	ACTCTCTTTT	TTCAGAAATG	CAAAGTAAAG	GTTTTTCAGG	GAAATCAAGA	CTCCTTCACA	7080
	CCTGTGGTGA	ACTCTCTAGA	CCCACCGTTA	CTGACTCGCT	ACCTTCGAAT	TCACCCCCAG	7140
5	AGTTGGGTGC	ACCAGATTGC	CCTGAGGATG	GAGGTTCTGG	GCTGCGAGGC	ACAGGACCTC	7200
	TACTGAGGGT	GGCCACTGCA	GCACCTGCCA	CTGCCGTCAC	CTCTCCCTCC	TCAGCTCCAG	7260
	GGCAGTGTCC	CTCCCTGGCT	TGCCCTTCTAC	CTTTGTGCTA	AATCCTAGCA	GACACTGCCT	7320
	TGAAGCCTCC	TGAATTAAC	ATCATCAGTC	CTGCATTTCT	TTGGTGGGGG	GCCAGGAGGG	7380
	TGCATCCAAT	TAACTTAAC	TCTTACCTAT	TTTCTGCAGC	TGCTCCCAGA	TTACTCCTTC	7440
10	CTTCCAATAT	AACTAGGCAA	AAAGAAAGTGA	GGAGAAACCT	GCATGAAAGC	ATTCTTCCCT	7500
	GAAAAGTTAG	GCCTCTCAGA	GTCACCACCT	CCTCTGTTGT	AGAAAACTA	TGTGATGAAA	7560
	CTTTGAAAAA	GATATTTATG	ATGTTAACAT	TTCAGGTTAA	GCCTCATACG	TTTAAATAA	7620
	AACTCTCAGT	TGTTTTATTAT	CCTGATCAAG	CATGGAACAA	AGCATGTTTC	AGGATCAGAT	7680
	CAATACAATC	TTGGAGTCAA	AAGGCAAATC	ATTTGGACAA	TCTGCAAAAT	GGAGAGAATA	7740
15	CAATAACTAC	TACAGTAAAG	TCTGTTTTCTG	CTTCCCTTACA	CATAGATATA	ATTATGTTAT	7800
	TTAGTCATTA	TGAGGGGCAC	ATTCTTATCT	CCAAAACCTAG	CATTCTTAAA	CTGAGAATTA	7860
	TAGATGGGGT	TCAAGAAATCC	CTAAGTCCCC	TGAAATTATA	TAAGGCATTC	TGTATAAATG	7920
	CAAATGTGCA	TTTTTCTGAC	GAGTGTCCAT	AGATATAAAG	CCATTGGTCT	TAATTCTGAC	7980
	CAATAAAAAA	ATAAGTCAGG	AGGATGCAAT	TGTTGAAAGC	TTTGAAATAA	AATAACATGT	8040
20	CTTCTTGAAA	TTTGTGATGG	CCAAGAAAGA	AAATGATGAT	GACATTAGGC	TTCTAAAGGA	8100
	CATACATTTA	ATATTTCTGT	GGAAATATGA	GGAAAATCCA	TGGTTATCTG	AGATAGGAGA	8160
	TACAAACTTT	GTAATTCTAA	TAATGCACTC	AGTTTACTCT	CTCCCTCTAC	TAATTTCTCTG	8220
	CTGAAAATAA	CACAACAAAA	ATGTAACAGG	GGAAATTATA	TACCGTGACT	GAAAACCTAGA	8280
	GTCCTACTTA	CATAGTTGAA	ATATCAAGGA	GGTCAGAAGA	AAATTGGACT	GGTGAAAACA	8340
25	GAAAAAACAC	TCCAGTCTGC	CATATCACCA	CACAATAGGA	TCCCCCTTCT	TGCCCTCCAC	8400
	CCCCATAAGA	TTGTGAAGGG	TTTACTGCTC	CTTCCATCTG	CCTGCACCCC	TTCACTATGA	8460
	CTACACAGAA	CTCTCCTGAT	AGTAAAGGGG	GCTGGAGGCA	AGGATAAGTT	ATAGAGCAGT	8520
	TGGAGGAAGC	ATCCAAAGAC	TGCAACCCAG	GGCAAATGGA	AAACAGGAGA	TCCTAATATG	8580
	AAAGAAAAAT	GGATCCCAAT	CTGAGAAAAG	GCAAAAGAAT	GGCTACTTTT	TTCTATGCTG	8640
30	GAGTATTTTC	TAATAATCCT	GCTTGACCCT	TATCTGACCT	CTTTGGAAAC	TATAACATAG	8700
	CTGTCACAGT	ATAGTCACAA	TCCACAAATG	ATGCAGGTGC	AAATGGTTTA	TAGCCCTGTG	8760
	AAGTTCTTAA	AGTTTAGAGG	CTAACCTTACA	GAAATGAATA	AGTTGTTTTG	TTTTATAGCC	8820
	CGGTAGAGGA	GTAAACCCCA	AAGGTGATAT	GGTTTTATTT	CCTGTTATGT	TTAACTTGAT	8880
	AATCTTATTT	TGGCATTTCT	TTCCCATTTGA	CTATATACAT	CTCTATTTCT	CAAATGTTCA	8940
35	TGGAACCTAGC	TCTTTTATTT	TCTTGCTGGT	TTCTTCAGTA	ATGAGTTAAA	TAAAACATTG	9000
	ACACATACA						9009

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8575 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

SUBSTITUTE SHEET (RULE 26)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

	GCAGCTGAGA	GCATGGCCTA	GGGTGGGCGG	CACCATTGTC	CAGCAGCTGA	GTITCCCAGG	60
5	GACCTTGGAG	ATAGCCGCAG	CCCTCATTTG	CAGGGGAAGA	TGATTCCTGC	CAGATTTGCC	120
	GGGGTGCTGC	TTGCTCTGGC	CCTCATTTTG	CCAGGGACCC	TTTGTGCAGA	AGGAACTCGC	180
	GGCAGGTCAT	CCACGGCCCC	ATGCAGCCTT	TTCGGAAGTG	ACTTCGTCAA	CACCTTTGAT	240
	GGGAGCATGT	ACAGCTTTGC	GGGATACTGC	AGTTACCTCC	TGGCAGGGGG	CTGCCAGAAA	300
	CGCTCCTTCT	CGATTATTGG	GGACTTCCAG	AATGGCAAGA	GAGTGAGCCT	CTCCGTGTAT	360
10	CTTGGGGAAT	TTTTTGACAT	CCATTTGTTT	GTCAATGGTA	CCGTGACACA	GGGGGACCAA	420
	AGAGTCTCCA	TGCCCTATGC	CTCCAAAGGG	CTGTATCTAG	AAACTGAGGC	TGGGTACTAC	480
	AAGCTGTCCG	GTGAGGCCTA	TGGCTTTGTG	GCCAGGATCG	ATGGCAGCGG	CAACTTTCAA	540
	GTCCTGCTGT	CAGACAGATA	CTTCAACAAG	ACCTGCGGGC	TGTGTGGCAA	CTTTAACATC	600
	TTTGCTGAAG	ATGACTTTAT	GACCCAAGAA	GGGACCTTGA	CCTCGGACCC	TTATGACTTT	660
15	GCCAACTCAT	GGGCTCTGAG	CAGTGGAGAA	CAGTGGTGTG	AACGGGCATC	TCCTCCCAGC	720
	AGCTCATGCA	ACATCTCCTC	TGGGGAAATG	CAGAAGGGCC	TGTGGGAGCA	GTGCCAGCTT	780
	CTGAAGAGCA	CCTCGGTGTT	TGCCCGCTGC	CACCCTCTGG	TGGACCCCGA	GCCTTTTGTG	840
	GCCCTGTGTG	AGAAGACTTT	GTGTGAGTGT	GCTGGGGGGC	TGGAGTGCGC	CTGCCCTGCC	900
	CTCCTGGAGT	ACGCCCCGAC	CTGTGCCCAG	GAGGGAATGG	TGCTGTACGG	CTGGACCGAC	960
20	CACAGCGCGT	GCAGCCCAGT	GTGCCCTGCT	GGTATGGAGT	ATAGGCAGTG	TGTGTCCCCT	1020
	TGCGCCAGGA	CCTGCCAGAG	CCTGCACATC	AATGAAATGT	GTCAGGAGCG	ATGCGTGGAT	1080
	GGCTGCAGCT	GCCCTGAGGG	ACAGCTCCTG	GATGAAGGCC	TCTGCGTGGA	GAGCACCGAG	1140
	TGTCCCTGCG	TGCATTCCGG	AAAGCGCTAC	CCTCCCGGCA	CCTCCCTCTC	TCGAGACTGC	1200
	AACACCTGCA	TTTGCCGAAA	CAGCCAGTGG	ATCTGCAGCA	ATGAAGAATG	TCCAGGGGAG	1260
25	TGCCTTGTCA	CAGGTCAATC	ACACTTCAAG	AGCTTTGACA	ACAGATACTT	CACCTTCAGT	1320
	GGGATCTGCC	AGTACCTGCT	GGCCCGGGAT	TGCCAGGACC	ACTCCTTCTC	CATTGTCAAT	1380
	GAGACTGTCC	AGTGTGCTGA	TGACCGCGAC	GCTGTGTGCA	CCCGCTCCGT	CACCGTCCGG	1440
	CTGCCTGGCC	TGCACAACAG	CCTTGTGAAA	CTGAAGCATG	GGGCAGGAGT	TGCCATGGAT	1500
	GGCCAGGACG	TCCAGCTCCC	CCTCCTGAAA	GGTGACCTCC	GCATCCAGCA	TACAGTGACG	1560
30	GCCTCCGTGC	GCCTCAGCTA	CGGGGAGGAC	CTGCAGATGG	ACTGGGATGG	CCGCGGGAGG	1620
	CTGCTGGTGA	AGCTGTCCCC	CGTCTATGCC	GGGAAGACCT	GCGGCCTGTG	TGGGAATTAC	1680
	AATGGCAACC	AGGGCGACGA	CTTCCTTACC	CCCTCTGGGC	TGGCGGAGCC	CCGGGTGGAG	1740
	GACTTCGGGA	ACGCTTGGA	GCTGCACGGG	GACTGCCAGG	ACCTGCAGAA	GCAGCACAGC	1800
	GATCCCTGCG	CCCTCAACCC	GCGCATGACC	AGGTTCTCCG	AGGAGGCGTG	CGCGGTCTCTG	1860
35	ACGTCCCCCA	CATTGAGGGC	CTGCCATCGT	GCCGTGAGCC	CGCTGCCCTA	CCTGCGGAAC	1920
	TGCCGCTACG	ACGTGTGCTC	CTGCTCGGAC	GGCCGCGAGT	GCCTGTGCGG	CGCCCTGGCC	1980
	AGCTATGCCG	CGGCCTGCGC	GGGGAGAGGC	GTGCGCGTCG	CGTGGCGCGA	GCCAGGCCGC	2040
	TGTGAGCTGA	ACTGCCCCGA	AGGCCAGGTG	TACCTGCAGT	GCGGGACCCC	CTGCAACCTG	2100
	ACCTGCCGCT	CTCTCTCTTA	CCCGGATGAG	GAATGCAATG	AGGCCTGCCT	GGAGGGCTGC	2160
40	TTCTGCCCCC	CAGGGCTCTA	CATGGATGAG	AGGGGGGACT	GCGTGCCCAA	GGCCCAGTGC	2220
	CCCTGTTACT	ATGACGGTGA	GATCTTCCAG	CCAGAAGACA	TCTTCTCAGA	CCATCACACC	2280
	ATGTGCTACT	GTGAGGATGG	CTTCATGCAC	TGTACCATGA	GTGGAGTCCC	CGGAAGCTTG	2340
	CTGCCTGACG	CTGTCTCAG	CAGTCCCCTG	TCTCATCGCA	GCAAAAGGAG	CCTATCCTGT	2400
	CGGCCCCCCA	TGGTCAAGCT	GGTGTGTCCC	GCTGACAACC	TGCGGGCTGA	AGGGCTCGAG	2460
45	TGTACCAAAA	CGTGCCAGAA	CTATGACCTG	GAGTGCATGA	GCATGGGCTG	TGTCTCTGGC	2520
	TGCCTCTGCC	CCCCGGGCAT	GGTCCGGCAT	GAGAACAGAT	GTGTGGCCCT	GGAAAGGTGT	2580
	CCCTGCTTCC	ATCAGGGCAA	GGAGTATGCC	CCTGGAGAAA	CAGTGAAGAT	TGGCTGCAAC	2640

SUBSTITUTE SHEET (RULE 26)

	ACTTGTGTCT	GTCGGGACCG	GAAGTGG AAC	TGCACAGACC	ATGTGTGTGA	TGCCACGTGC	2700
	TCCACGATCG	GCATGGCCCA	CTACCTCACC	TTCCGACGGC	TCAAATACCT	GTTCCCCGGG	2760
	GAGTGCCAGT	ACGTTCTGGT	GCAGGATTAC	TGCGGCAGTA	ACCCTGGGAC	CTTTCGGATC	2820
	CTAGTGGGGA	ATAAGGGATG	CAGCCACCCC	TCAGTGAAAT	GCAAGAAACG	GGTCACCATC	2880
5	CTGGTGGAGG	GAGGAGAGAT	TGAGCTGTTT	GACGGGGAGG	TGAATGTGAA	GAGGCCCCATG	2940
	AAGGATGAGA	CTCACTTTGA	GGTGGTGGAG	TCTGGCCGGT	ACATCATTTCT	GCTGCTGGGC	3000
	AAAGCCCTCT	CCGTGGTCTG	GGACCGCCAC	CTGAGCATCT	CCGTGGTCCT	GAAGCAGACA	3060
	TACCAGGAGA	AAGTGTGTGG	CCTGTGTGGG	AATTTTGATG	GCATCCAGAA	CAATGACCTC	3120
	ACCAGCAGCA	ACCTCCAAGT	GGAGGAAGAC	CCTGTGGACT	TTGGGAACTC	CTGGAAAGTG	3180
10	AGCTCGCAGT	GTGCTGACAC	CAGAAAAGTG	CCTCTGGACT	CATCCCCCTGC	CACCTGCCAT	3240
	AACAACATCA	TGAAGCAGAC	GATGGTGGAT	TCCTCCTGTA	GAATCCTTAC	CAGTGACGTC	3300
	TTCCAGGACT	GCAACAAGCT	GGTGGACCCC	GAGCCATATC	TGGATGTCTG	CATTTACGAC	3360
	ACCTGCTCCT	GTGAGTCCAT	TGGGGACTGC	GCCTGCTTCT	GCGACACCAT	TGCTGCCTAT	3420
	GCCCCAGTGT	GTGCCCAGCA	TGGCAAGGTG	GTGACCTGGA	GGACGGCCAC	ATTGTGCCCC	3480
15	CAGAGCTGCG	AGGAGAGGAA	TCTCCGGGAG	AACGGGTATG	AGTGTGAGTG	GCGCTATAAC	3540
	AGCTGTGCAC	CTGCCGTGTA	AGTCACGTGT	CAGCACCTTG	AGCCACTGGC	CTGCCCTGTG	3600
	CAGTGTGTGG	AGGGCTGCCA	TGCCCACTGC	CCTCCAGGGA	AAATCCTGGA	TGAGCTTTTG	3660
	CAGACCTGCG	TTGACCCTGA	AGACTGTCCA	GTGTGTGAGG	TGGCTGGCCG	GCGTTTTGCC	3720
	TCAGGAAAGA	AAGTCACCTT	GAATCCCAGT	GACCTGAGC	ACTGCCAGAT	TTGCCACTGT	3780
20	GATGTTGTCA	ACCTCACCTG	TGAAGCCTGC	CAGGAGCCGG	GAGGCCCTGGT	GGTGCCCTCCC	3840
	ACAGATGCCC	CGGTGAGCCC	CACCACTCTG	TATGTGGAGG	ACATCTCGGA	ACCGCCGTTG	3900
	CACGATTTCT	ACTGCAGCAG	GCTACTGGAC	CTGGTCTTCC	TGCTGGATGG	CTCCTCCAGG	3960
	CTGTCCGAGG	CTGAGTTTGA	AGTGCTGAAG	GCCTTTGTGG	TGGACATGAT	GGAGCGGCTG	4020
	CGCATCTCCC	AGAAGTGGGT	CCGCGTGGCC	GTGGTGGAGT	ACCACGACGG	CTCCCACGCC	4080
25	TACATCGGGC	TCAAGGACCG	GAAGCGACCG	TCAGAGCTGC	GGCGCATTGC	CAGCCAGGTG	4140
	AAGTATGCGG	GCAGCCAGGT	GGCCTCCACC	AGCGAGGTCT	TGAAATACAC	ACTGTTCCAA	4200
	ATCTTCAGCA	AGATCGACCG	CCCTGAAGCC	TCCCGCATCG	CCCTGCTCCT	GATGGCCAGC	4260
	CAGGAGCCCC	AACGGATGTC	CCGGAACTTT	GTCCGCTACG	TCCAGGGCCT	GAAGAAGAAG	4320
	AAGGTCATTG	TGATCCCGGT	GGGCATTGGG	CCCCATGCCA	ACCTCAAGCA	GATCCGCCTC	4380
30	ATCGAGAAGC	AGGCCCCCTGA	GAACAAGGCC	TTCTGTCTGA	GCAGTGTGGA	TGAGCTGGAG	4440
	CAGCAAAGGG	ACGAGATCGT	TAGCTACCTC	TGTGACCTTG	CCCCTGAAGC	CCCTCCTCCT	4500
	ACTCTGCCCC	CCCACATGGC	ACAAGTCACT	GTGGGCCCGG	GGCTCTTGGG	GGTTTCGACC	4560
	CTGGGGCCCA	AGAGGAACTC	CATGGTTCTG	GATGTGGCGT	TCGTCTTGGA	AGGATCGGAC	4620
	AAAATTGGTG	AAGCCGACTT	CAACAGGAGC	AAGGAGTTCA	TGGAGGAGGT	GATTTCAGCG	4680
35	ATGGATGTGG	GCCAGGACAG	CATCCACGTC	ACGGTGTCTG	AGTACTCCTA	CATGGTGACC	4740
	GTGGAGTACC	CCTTCAGCGA	GGCACAGTCC	AAAGGGGACA	TCCTGCAGCG	GGTGCGAGAG	4800
	ATCCGCTACC	AGGGCGGCAA	CAGGACCAAC	ACTGGGCTGG	CCCTGCGGTA	CCTCTCTGAC	4860
	CACAGCTTCT	TGGTCAGCCA	GGGTGACCGG	GAGCAGGCGC	CCAACCTGGT	CTACATGGTC	4920
	ACCGGAAATC	CTGCCTCTGA	TGAGATCAAG	AGGCTGCCTG	GAGACATCCA	GGTGGTGCCC	4980
40	ATTGGAGTGG	GCCCTAATGC	CAACGTGCAG	GAGCTGGAGA	GGATTGGCTG	GCCCAATGCC	5040
	CCTATCCTCA	TCCAGGACTT	TGAGACGCTC	CCCCGAGAGG	CTCCTGACCT	GGTGCTGCAG	5100
	AGGTGCTGCT	CCGGAGAGGG	GCTGCAGATC	CCCACCTCT	CCCCTGCACC	TGACTGCAGC	5160
	CAGCCCCCTG	ACGTGATCCT	TCTCCTGGAT	GGCTCCTCCA	GTTCCTCCAGC	TTCTTATTTT	5220
	GATGAAATGA	AGAGTTTTCG	CAAGGCTTTC	ATTTCAAAG	CCAATATAGG	GCCTCGTCTC	5280
45	ACTCAGGTGT	CAGTGCTGCA	GTATGGAAGC	ATCACCACCA	TTGACGTGCC	ATGGAACGTG	5340
	GTCCCGGAGA	AAGCCCATT	GCTGAGCCTT	GTGGACGTCA	TGCAGCGGGA	GGGAGGCCCC	5400
	AGCCAAATCG	GGGATGCCTT	GGGCTTTGCT	GTGCGATACT	TGACTTCAGA	AATGCATGGT	5460

SUBSTITUTE SHEET (RULE 26)

	GCCAGGCCGG	GAGCCTCAAA	GGCGGTGGTC	ATCCTGGTCA	CGGACGTCTC	TGTGGATTCA	5520
	GTGGATGCAG	CAGCTGATGC	CGCCAGGTCC	AACAGAGTGA	CAGTGTTCCT	TATTGGAATT	5580
	GGAGATCGCT	ACGATGCAGC	CCAGCTACGG	ATCTTGGCAG	GCCCAGCAGG	CGACTCCAAC	5640
	GTGGTGAAGC	TCCAGCGAAT	CGAAGACCTC	CCTACCATGG	TCACCTTGGG	CAATTCTTC	5700
5	CTCCACAAAC	TGTGCTCTGG	ATTTGTTAGG	ATTTGCATGG	ATGAGGATGG	GAATGAGAAG	5760
	AGGCCCCGGG	ACGTCTGGAC	CTTGCCAGAC	CAGTGCCACA	CCGTGACTTG	CCAGCCAGAT	5820
	GGCCAGACCT	TGCTGAAGAC	TCATCGGGTC	AACTGTGACC	GGGGGCTGAG	GCCTTCGTGC	5880
	CCTAACAGCC	AGTCCCCGTG	TAAAGTGGAA	GAGACCTGTG	GCTGCCGCTG	GACCTGCCCC	5940
	TGCGTGTGCA	CAGGCAGCTC	CACTCGGCAC	ATCGTGACCT	TTGATGGGCA	GAATTTCAAG	6000
10	CTGACTGGCA	GCTGTTCTTA	TGTCTATTT	CAAAACAAGG	AGCAGGACCT	GGAGGTGATT	6060
	CTCCATAATG	GTGCCCTGCAG	CCCTGGAGCA	AGGCAGGGCT	GCATGAAATC	CATCGAGGTG	6120
	AAGCACAGTG	CCCTCTCCGT	CGAGCTGCAC	AGTGACATGG	AGGTGACGGT	GAATGGGAGA	6180
	CTGGTCTCTG	TTCCTTACGT	GGGTGGGAAC	ATGGAAGTCA	ACGTTTATGG	TGCCATCATG	6240
	CATGAGGTCA	GATTCAATCA	CCTTGGTTCAC	ATCTTCACAT	TCACTCCACA	AAACAATGAG	6300
15	TTCCAACCTG	AGCTCAGCCC	CAAGACTTTT	GCTTCAAAGA	CGTATGGTCT	GTGTGGGATC	6360
	TGTGATGAGA	ACGGAGCCAA	TGACTTCATG	CTGAGGGATG	GCACAGTCAC	CACAGACTGG	6420
	AAAACACTTG	TTCAGGAATG	GACTGTGCAG	CGGCCAGGGC	AGACGTGCCA	GCCCATCCTG	6480
	GAGGAGCAGT	GTCTTGTCCC	CGACAGCTCC	CACTGCCAGG	TCCTCCTCTT	ACCACTGTTT	6540
	GCTGAATGCC	ACAAGGTCCT	GGCTCCAGCC	ACATTCTATG	CCATCTGCCA	GCAGGACAGT	6600
20	TGCCACCAGG	AGCAAGTGTG	TGAGGTGATC	GCCTCTTATG	CCCACCTCTG	TCCGACCAAC	6660
	GGGGTCTGCG	TTGACTGGAG	GACACCTGAT	TTCTGTGCTA	TGTCATGCCC	ACCATCTCTG	6720
	GTCTACAACC	ACTGTGAGCA	TGGCTGTCCC	CGGCACTGTG	ATGGCAACGT	GAGCTCCTGT	6780
	GGGGACCATC	CCTCCGAAGG	CTGTTTCTGC	CCTCCAGATA	AAGTCATGTT	GGAAGGCAGC	6840
	TGTGTCCCTG	AAGAGGCCTG	CACTCAGTGC	ATTGGTGAGG	ATGGAGTCCA	GCACCAGTTC	6900
25	CTGGAAGCCT	GGGTCCCGGA	CCACCAGCCC	TGTCAGATCT	GCACATGCCT	CAGCGGGCGG	6960
	AAGGTCAACT	GCACAACGCA	GCCCTGCCCC	ACGGCCAAAG	CTCCCACGTG	TGGCCTGTGT	7020
	GAAGTAGCCC	GCCTCCGCCA	GAATGCAGAC	CAGTGCTGCC	CCGAGTATGA	GTGTGTGTGT	7080
	GACCCAGTGA	GCTGTGACCT	GCCCCCAGTG	CCTCACTGTG	AACGTGGCCT	CCAGCCCACA	7140
	CTGACCAACC	CTGGCGAGTG	CAGACCCAAC	TTCACCTGCG	CCTGCAGGAA	GGAGGAGTGC	7200
30	AAAAGAGTGT	CCCCACCCTC	CTGCCCCCCG	CACCGTTTGC	CCACCCTTCG	GAAGACCCAG	7260
	TGCTGTGATG	AGTATGAGTG	TGCCTGCAAC	TGTGTCAACT	CCACAGTGAG	CTGTCCCCTT	7320
	GGGTACTTGG	CCTCAACCGC	CACCAATGAC	TGTGGCTGTA	CCACAACCAC	CTGCCTTCCC	7380
	GACAAGGTGT	GTGTCCACCG	AAGCACCATC	TACCTGTGG	GCCAGTTCTG	GGAGGAGGGC	7440
	TGCGATGTGT	GCACCTGCAC	CGACATGGAG	GATGCCGTGA	TGGGCCTCCG	CGTGGCCCAG	7500
35	TGCTCCCAGA	AGCCCTGTGA	GGACAGCTGT	CGGTCCGGCT	TCACCTACGT	TCTGCATGAA	7560
	GGCGAGTGCT	GTGGAAGGTG	CCTGCCATCT	GCCTGTGAGG	TGGTGACTGG	CTCACCOCGG	7620
	GGGGACTCCC	AGTCTTCCTG	GAAGAGTGTG	GGCTCCAGT	GGGCCTCCCC	GGAGAACCCC	7680
	TGCCTCATCA	ATGAGTGTGT	CCGAGTGAAG	GAGGAGGTCT	TTATACAACA	AAGGAACGTC	7740
	TCCTGCCCCC	AGCTGGAGGT	CCCTGTCTGC	CCCTCGGGCT	TTCAGCTGAG	CTGTAAGACC	7800
40	TCAGCGTGCT	GCCCAAGCTG	TCGCTGTGAG	CGCATGGAGG	CCTGCATGCT	CAATGGCACT	7860
	GTCATTGGGC	CCGGGAAGAC	TGTGATGATC	GATGTGTGCA	CGACCTGCCG	CTGCATGGTG	7920
	CAGGTGGGGG	TCATCTCTGG	ATTCAAGCTG	GAGTGCAGGA	AGACCACCTG	CAACCCCTGC	7980
	CCCCTGGGTT	ACAAGGAAGA	AAATAACACA	GGTGAATGTT	GTGGGAGATG	TTTGCCTACG	8040
	GCTTGCACCA	TTCAGCTAAG	AGGAGGACAG	ATCATGACAC	TGAAGCGTGA	TGAGACGCTC	8100
45	CAGGATGGCT	GTGATACTCA	CTTCTGCAAG	GTCAATGAGA	GAGGAGAGTA	CTTCTGGGAG	8160
	AAGAGGGTCA	CAGGCTGCCC	ACCCCTTTGAT	GAACACAAGT	GTCTGGCTGA	GGGAGGTAAA	8220
	ATTATGAAAA	TTCCAGGCAC	CTGCTGTGAC	ACATGTGAGG	AGCCTGAGTG	CAACGACATC	8280

SUBSTITUTE SHEET (RULE 26)

ACTGCCAGGC TGCAGTATGT CAAGGTGGGA AGCTGTAAGT CTGAAGTAGA GGTGGATATC 8340
 CACTACTGCC AGGGCAAATG TGCCAGCAA GCCATGTACT CCATTGACAT CAACGATGTG 8400
 CAGGACCAGT GCTCCTGCTG CTCTCCGACA CGGACGGAGC CCATGCAGGT GGCCCTGCAC 8460
 TGCACCAATG GCTCTGTTGT GTACCATGAG GTTCTCAATG CCATGGAGTG CAAATGCTCC 8520
 5 CCCAGGAAGT GCAGCAAGTG AGGCTGCTGC AGCTGCATGG GTGCCTGCTG CTGCC 8575

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 13815 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

20 CCTCACTCAC TTGCCCCCTTA CAGGACTCAG CTCTTGAAGG CAATAGCTTT ATAGAAAAAA 60
 CGAATAGGAA GACTTGAAGT GCTATTTTTT TTTTTTTTTT TGTCAAGGCT GCTGAAGTTT 120
 ATTGGCTTCT CATCGTACCT AAGCCTCCTG GAGCAATAAA ACTGGGAGAA ACTTTTACCA 180
 AGATTTTTAT CCCTGCCTTG ATATATACTT TTTCTTCCAA ATGCTTTGGT GGGAAGAAGT 240
 25 AGAGGACTGT TATGAAAGAG AAGATGTTCA AAAGAAAACA TTCACAAAAT GGATAAATGC 300
 ACAATTTTCT AAGTTTGGAA AGCAACACAT AGACAACCTC TTCAGTGACC TGCAGGATGG 360
 AAAACGCCTC CTAGACCTCT TGGAAGGCCT TACAGGGCAA AAAGTCCAA AAGAAAAGGG 420
 ATCTACAAGA GTTCATGCCC TGAACAATGT CAACAAGGCA CTGCGGGTCT TACAGAAAAA 480
 TAATGTTGAT TTAGTGAATA TAGGAAGCAC TGACATAGTG GATGGAAATC ATAAACTCAC 540
 30 TCTTGGTTTG ATTTGGAATA TAATCCTCCA CTGGCAGGTC AAAAATGTGA TGAAAACAT 600
 CATGGCTGGA TTGCAGCAA CCAACAGTGA AAAGATTCTT CTGAGCTGGG TTCGACAGTC 660
 AACACGTAAT TATCCACAGG TTAACGTCAT CAACTTCACC TCTAGCTGGT CCGACGGGTT 720
 GGCTTTGAAT GCTCTTATCC ATAGTCACAG GCCCGACCTG TTTGATTGGA ATAGTGTGGT 780
 TTCACAGCAC TCAGCCACCC AAAGACTGGA ACATGCCTTC AACATTGCAA AATGCCAGTT 840
 35 AGGCATAGAA AAACCTCTTG ATCCTGAAGA TGTTGCTACC ACTTATCCAG ACAAGAAATC 900
 CATCTTAATG TACATCACAT CACTCTTTCA AGTTTTGCCA CAACAAGTGA GCATTGAAGC 960
 CATTCAAGAA GTGGAAATGT TGCCAGGAC ATCTTCAAAA GTAAC TAGAG AAGAACATTT 1020
 TCAATTACAT CACCAGATGC ATTACTCTCA ACAGATCACA GTCAGTCTAG CACAGGGCTA 1080
 TGAACAAACT TCTTCATCTC CTAAGCCTCG ATTCAAGAGT TATGCCITCA CACAGGCTGC 1140
 40 TTATGTTGCC ACCTCTGATT CCACACAGAG CCCCTATCCT TCACAGCATT TGGAAGCTCC 1200
 CAGAGACAAG TCACTTGACA GTTCATTGAT GGAGACGGAA GTAAATCTGG ATAGTTACCA 1260
 AACTGCTTTA GAAGAAGTAC TTTTATGGCT TCTTTCTGCC GAGGATACAT TGCGAGCACA 1320
 AGGAGAGATT TCAAATGATG TTGAAGAAGT GAAAGAACAG TTTTATGCTC ATGAGGGATT 1380
 CATGATGGAT CTGACATCTC ATCAAGGACT TGTTGGTAAT GTTCTACAGT TAGGAAGTCA 1440
 45 ACTAGTTGGA AAAGGGAAAT TATCAGAAGA TGAAGAAGCT GAAGTGCAAG AACAAATGAA 1500
 TCTCCTAAAT TCAAGATGGG AATGTCTCAG GGTAGCTAGC ATGGAAAAAC AAAGCAAATT 1560
 ACACAAAGTT CTAATGGATC TCCAGAATCA GAAATTAAAA GAACTAGATG ACTGGTTAAC 1620

SUBSTITUTE SHEET (RULE 26)

	AAAACTGAA	GAGAGAACTA	AGAAAATGGA	GGAAGAGCCC	TTTGGACCTG	ATCTTGAAGA	1680
	TCTAAAATGC	CAAGTACAAC	AACATAAGGT	GCTTCAAGAA	GATCTAGAAC	AGGAGCAGGT	1740
	CAGGGTCAAC	TCGCTCACTC	ACATGGTAGT	AGTGGTTGAT	GAATCCAGCG	GTGATCATGC	1800
	AACAGCTGCT	TTGGAAGAAC	AACTTAAGGT	ACTGGGAGAT	CGATGGGCAA	ATATCTGCAG	1860
5	ATGGACTGAA	GACCGCTGGA	TTGTTTTTACA	AGATATTCTT	CTAAAATGGC	AGCATTTTAC	1920
	TGAAGAACAG	TGCCTTTTTTA	GTACATGGCT	TTTCAGAAAA	GAAGATGCAA	TGAAGAACAT	1980
	TCAGACAAGT	GGCTTTTAAAG	ATCAAAATGA	AATGATGTCA	AGTCTTCACA	AAATATCTAC	2040
	TTTAAAAATA	GATCTAGAAA	AGAAAAAGCC	AACCATGGAA	AAACTAAGTT	CACTCAATCA	2100
	AGATCTACTT	TCGGCACTGA	AAAATAAGTC	AGTGAICTAA	AAGATGGAAA	TCTGGATGGA	2160
10	AAACTTTGCA	CAACGTTGGG	ACAATTTAAC	CCAAAACTT	GAAAAGAGTT	CAGCACAAAT	2220
	TTCACAGGCT	GTCACCACCA	CTCAACCATC	CCTAACACAG	ACAACGTGTA	TGGAAACGGT	2280
	AACTATGGTG	ACCACAAGGG	AACAAATCAT	GGTAAAACAT	GCCCAAGAGG	AACTTCCACC	2340
	ACCACCTCCT	CAAAAGAAGA	GGCAGATAAC	TGTGGATTCT	GAACTCAGGA	AAAGGTTGGA	2400
	TGTCGATATA	ACTGAACTTC	ACAGTTGGAT	TACTCGTTCA	GAAGCTGTAT	TACAGAGTTC	2460
15	TGAATTTGCA	GTCTATCGAA	AAGAAGGCAA	CATCTCAGAC	TTGCAAGAAA	AAGTCAATGC	2520
	CATAGCACGA	GAAAAAGCAG	AGAAGTTCAG	AAAAC TGCA	GATGCCAGCA	GATCAGCTCA	2580
	GGCCCTGGTG	GAACAGATGG	CAAATGAGGG	TGTTAATGCT	GAAAGTATCA	GACAAGCTTC	2640
	AGAACAAC TG	AACAGCCGGT	GGACAGAATT	CTGCCAATTG	CTGAGTGAGA	GAGTTAACTG	2700
	GCTAGAGTAT	CAAACCAACA	TCATTACCTT	TTATAATCAG	CTACAACAAT	TGGAACAGAT	2760
20	GACAACTACT	GCCGAAAACT	TGTTGAAAAC	CCAGTCTACC	ACCCTATCAG	AGCCAACAGC	2820
	AATTAAAGC	CAGTTAAAAA	TTTGTAAGGA	TGAAGTCAAC	AGATTGTCAG	CTCTTCAGCC	2880
	TCAAATTGAG	CAATTAAAAA	TTCAGAGTCT	ACAAC TGAA	GAAAAGGGAC	AGGGGCCAAT	2940
	GTTTTCTGGAT	GCAGACTTTG	TGGCCTTTAC	TAATCATTTT	AACCACATCT	TTGATGGTGT	3000
	GAGGGCCAAA	GAGAAAGAGC	TACAGACAAT	TTTTTGACACT	TTACCACCAA	TGCGCTATCA	3060
25	GGAGACAATG	AGTAGCATCA	GGACGTGGAT	CCAGCAGTCA	GAAAGCAAAC	TCTCTGTACC	3120
	TTATCTTAGT	GTTACTGAAT	ATGAAATAAT	GGAGGAGAGA	CTCGGGAAAT	TACAGGCTCT	3180
	GCAAAGTTCT	TTGAAAGAGC	AACAAAATGG	CTTCAACTAT	CTGAGTGACA	CTGTGAAGGA	3240
	GATGGCCAAG	AAAGCACCTT	CAGAAATATG	CCAGAAATAT	CTGTCAGAAT	TTGAAGAGAT	3300
	TGAGGGGCAC	TGGAAGAAAC	TTTCCTCCCA	GTTGGTGGAA	AGCTGCCAAA	AGCTAGAAGA	3360
30	ACATATGAAT	AAACTTCGAA	AATTTTCAGAA	TCACATAAAA	ACCTTACAGA	AATGGATGGC	3420
	TGAAGTTGAT	GTTTTCTCTGA	AAGAGGAATG	GCCTGCCCTG	GGGGATGCTG	AAATCCTGAA	3480
	AAAACAGCTC	AAACAATGCA	GACTTTT TAGT	TGGTGATATT	CAAACAATTC	AGCCCAGTTT	3540
	AAATAGTGTT	AATGAAGGTG	GGCAGAAGAT	AAAGAGTGAA	GCTGAACTTG	AGTTTGCATC	3600
	CAGACTGGAG	ACAGAACTTA	GAGAGCTTAA	CACTCAGTGG	GATCACATAT	GCCGCCAGGT	3660
35	CTACACCAGA	AAGGAAGCCT	TAAAGGCAGG	TTTGGATAAA	ACCGTAAGCC	TCCAAAAAGA	3720
	TCTATCAGAG	ATGCATGAGT	GGATGACACA	AGCTGAAGAA	GAATATCTAG	AGAGAGATTT	3780
	TGAATATAAA	ACTCCAGATG	AATTACAGAC	TGCTGTTGAA	GAAATGAAGA	GAGCTAAAGA	3840
	AGAGGCACTA	CAAAAAGAAA	CTAAAGTGAA	ACTCCTTACT	GAGACTGTAA	ATAGTGTAAT	3900
	AGCTCACGCT	CCACCCTCAG	CACAAGAGGC	CTTAAAAAAG	GAACTTGAAA	CTCTGACCAC	3960
40	CAACTACCAA	TGGCTGTGCA	CCAGGCTGAA	TGGAAAATGC	AAAAC TTTGG	AAGAAGTTTG	4020
	GGCATGTTGG	CATGAGTTAT	TGTCATATTT	AGAGAAAAGCA	AACAAGTGGC	TCAATGAAGT	4080
	AGAATTGAAA	CTTAAAACCA	TGGAAAATGT	TCCTGCAGGA	CCTGAGGAAA	TCACTGAAGT	4140
	GCTAGAATCT	CTTGAAAATC	TGATGCATCA	TTCAGAGGAG	AACCCAAATC	AGATTCTGCT	4200
	ATTGGCACAG	ACTCTTACAG	ATGGAGGAGT	CATGGATGAA	CTGATCAATG	AGGAGCTTGA	4260
45	GACGTTTAAAT	TCTCGTTTGA	GGGAACTACA	TGAAGAGGCT	GTGAGGAAAC	AAAAGTTGCT	4320
	TGAACAGAGT	ATCCAGTCTG	CCCAGGAAAT	TGAAAAGTCC	TTGCACTTAA	TTCAGGAGTC	4380
	GCTTGAATTC	ATTGACAAGC	AGTTGGCAGC	TTATATCACT	GACAAGGTGG	ATGCAGCTCA	4440

	AATGCCTCAG	GAAGCCCAGA	AAATCCAATC	AGATTTGACA	AGTCATGAGA	TAAGTTTAGA	4500
	AGAAATGAAG	AAACATAACC	AGGGGAAGGA	TGCCAACCAA	AGGGTTCTTT	CACAAATTGA	4560
	TGTTGCACAG	AAAAAATTAC	AAGATGTCTC	CATGAAATTT	CGATTATTCC	AAAAACCAGC	4620
	CAATTTTGAA	CAACGTCTAG	AGGAAAGTAA	GATGATTTTA	GATGAAGTCA	AGATGCATTT	4680
5	GCCTGCATTG	GAAACCAAGA	GTGTTGAACA	GGAAGTAATT	CAGTCACAAC	TAAGTCATTG	4740
	TGTGAACTTG	TATAAAAGCC	TGAGTGAAGT	CAAGTCTGAA	GTGGAAATGG	TGATTAAAAC	4800
	CGGACGTCAA	ATTGTACAGA	AAAAGCAGAC	AGAAAATCCC	AAAGAGCTTG	ATGAACGAGT	4860
	AACAGCTTTG	AAATTGCATT	ACAATGAGTT	GGGTGCGAAG	GTAACAGAGA	GAAAGCAACA	4920
	GTGGGAGAAA	TGCTTGAAGT	TGTCCCGTAA	GATGAGAAAG	GAAATGAATG	TCTTAACAGA	4980
10	ATGGCTGGCA	GCAACAGATA	CAGAATTGAC	GAAGAGATCA	GCAGTTGAAG	GAATGCCAAG	5040
	TAATTTGGAT	TCTGAAGTTG	CCTGGGGAAA	GGCTACTCAA	AAAGAGATTG	AGAAACAGAA	5100
	GGCTCACTTG	AAGAGTGTTA	CAGAATTAGG	AGAGTCTTTG	AAAATGGTGT	TGGGCAAGAA	5160
	AGAAACCTTG	GTAGAAGATA	AACTGAGTCT	TCTGAACAGT	AACTGGATAG	CTGTCACCTC	5220
	CAGAGTAGAA	GAATGGCTAA	ATCTTTTGTT	GGAATACCAG	AAACACATGG	AAACCTTTGA	5280
15	TCAGAACATA	GAACAAATCA	CAAAGTGGAT	CATTCATGCA	GATGAACTTT	TAGATGAGTC	5340
	TGAAAAGAAG	AAACCACAAC	AAAAGGAAGA	CATTCTTAAG	CGTTTAAAGG	CTGAAATGAA	5400
	TGACATGCGC	CCAAAGGTGG	ACTCCACACG	TGACCAAGCA	GCAAAATTGA	TGGCAAACCG	5460
	CGGTGACCAC	TGCAGGAAAG	TAGTAGAGCC	CCAAATCTCT	GAGCTCAACC	GTCCGATTTGC	5520
	AGCTATTTCT	CACAGAATTA	AGACTGGAAA	GGCCTCCATT	CCTTTGAAGG	AATTGGAGCA	5580
20	GTITTAECTCA	GATATACAAA	AATTGCTTGA	ACCACTGGAG	GCTGAAATTC	AGCAGGGGGT	5640
	GAATCTGAAA	GAGGAAGACT	TCAATAAAGA	TATGAGTGAA	GACAAATGAGG	GTACTGTAAA	5700
	TGAAITGTTG	CAAAGAGGAG	ACAACCTTACA	ACAAAGAATC	ACAGATGAGA	GAAAGCGAGA	5760
	GGAAATAAAG	ATAAAACAGC	AGCTGTTACA	GACAAAACAT	AATGCTCTCA	AGGATTTGAG	5820
	GTCTCAAAGA	AGAAAAAAGG	CCCTAGAAAT	TTCTCACCAG	TGGTATCAGT	ACAAGAGGCA	5880
25	GGCTGATGAT	CTCCTGAAAT	GCTTGGATGA	AATTGAAAAA	AAATTAGCCA	GCCTACCTGA	5940
	ACCCAGAGAT	GAAAGAAAAT	TAAAGGAAAT	TGATCGTGAA	TTGCAGAAGA	AGAAAGAGGA	6000
	GCTGAATGCA	GTGCGCAGGC	AAGCTGAGGG	CTTGTCTGAG	AATGGGGCCG	CAATGGCAGT	6060
	GGAGCCAACT	CAGATCCAGC	TCAGCAAGCG	CTGGCGGCAA	ATTGAGAGCA	ATTTTGCTCA	6120
	GTTTCGAAGA	CTCAACTTTG	CACAAATTCA	CACTCTCCAT	GAAGAAACTA	TGGTAGTGAC	6180
30	GACTGAAGAT	ATGCCTTTGG	ATGTTTCTTA	TGTGCCTTCT	ACTTATTTGA	CCGAGATCAG	6240
	TCATATCTTA	CAAGCTCTTT	CAGAAGTTGA	TCATCTTCTA	AATACTCCTG	AACTCTGTGC	6300
	TAAAGATTTT	GAAGATCTTT	TTAAGCAAGA	GGAGTCTCTT	AAGAATATAA	AAGACAATTT	6360
	GCAACAAATC	TCAGGTCGGA	TTGATATTAT	TCACAAGAAG	AAGACAGCAG	CCTTGCAAAG	6420
	TGCCACCTCC	ATGGAAAAGG	TGAAAGTACA	GGAAGCCGTG	GCACAGATGG	ATTTCCAGGG	6480
35	GGAAAAACTT	CATAGAATGT	ACAAGGAACG	ACAAGGGCGA	TTGACAGAT	CAGTTGAAAA	6540
	ATGGCGACAC	TTTCATTATG	ATATGAAGGT	ATTTAATCAA	TGGCTGAATG	AAGTTGAACA	6600
	GTTTTTCAAA	AAGACACAAA	ATCCTGAAAA	CTGGGAACAT	GCTAAATACA	AATGGTATCT	6660
	TAAGGAACTC	CAGGATGGCA	TTGGGCAGCG	TCAAGCTGTT	GTCAGAACAC	TGAATGCAAC	6720
	TGGGGAAGAA	ATAATTCAAC	AGTCTTCAAA	AACAGATGTC	AATATTCTAC	AAGAAAAATT	6780
40	AGGAAGCTTG	AGTCTGCGGT	GGCACGACAT	CTGCAAAGAG	CTGGCAGAAA	GGAGAAAGAG	6840
	GATTGAAGAA	CAAAAGAATG	TCTTGTGAGA	ATTTCAAAGA	GATTTAAATG	AATTTGTTTT	6900
	GTGGCTGGAA	GAAGCAGATA	ACATTGCTAT	TACTCCACTT	GGAGATGAGC	AGCAGCTAAA	6960
	AGAACAACCT	GAACAAGTCA	AGTTACTGGC	AGAAGAGTTG	CCCCTGCGCC	AGGGAATTCT	7020
	AAAACAATTA	AATGAAACAG	GAGGAGCAGT	ACTTGTAAGT	GCTCCCATAA	GGCCAGAAGA	7080
45	GCAAGATAAA	CTTGAAAAGA	AGCTCAAACA	GACAAATCTC	CAGTGGATAA	AGGTCTCCAG	7140
	AGCTTTACCT	GAGAAACAAG	GAGAGCTTGA	GGTTCACCTA	AAAGATTTTA	GGCAGCTTGA	7200
	AGAGCAGCTG	GATCACCTGC	TTCTGTGGCT	CTCTCCTATT	AGAAACCACT	TGGAAATTTA	7260

SUBSTITUTE SHEET (RULE 26)

	TAACCAACCA	AGTCAGGCAG	GACCGTTTGA	CATAAAGGAG	ATTGAAGTAA	CAGTTCACGG	7320
	TAAACAAGCG	GATGTGGAAA	GGCTTTTGTG	GAAAGGGCAG	CATTTGTATA	AGGAAAAACC	7380
	AAGCACTCAG	CCAGTGAAGA	GGAAGTTAGA	AGATCTGAGG	TCTGAGTGGG	AGGCTGTAAA	7440
	CCATTTACTT	CGGGAGCTGA	GGACAAAGCA	GCCTGACCGT	GCCCCTGGAC	TGAGCACTAC	7500
5	TGGAGCCTCT	GCCAGTCAGA	CTGTTACTCT	AGTGACACAA	TCTGTGGTTA	CTAAGGAAAC	7560
	TGTCATCTCC	AAACTAGAAA	TGCCATCTTC	TTTGCTGTGT	GAGGTACCTG	CACTGGCAGA	7620
	CTTCAACCGA	GCTTGGACAG	AACTTACAGA	CTGGCTGTCT	CTGCTTGATC	GAGTTATAAA	7680
	ATCACAGAGA	GTGATGGTGG	GTGATCTGGA	AGACATCAAT	GAAATGATCA	TCAAACAGAA	7740
	GGCAACACTG	CAAGATTTGG	AACAGAGACG	CCCCCAATTG	GAAGAACTCA	TTACTGCTGC	7800
10	CCAGAATTTG	AAAAACAAA	CCAGCAATCA	AGAAGCTAGA	ACAATCATTA	CTGATCGAAT	7860
	TGAAAGAATT	CAGATTCAGT	GGGATGAGGT	TCAAGAACAG	CTGCAGAACA	GGAGACAACA	7920
	GTTGAATGAA	ATGTTAAAGG	ATTCAACACA	ATGGCTGGAA	GCTAAGGAAG	AAGCCGAACA	7980
	GGTCATAGGA	CAGGTCAGAG	GCAAGCTTGA	CTCATGGAAA	GAAGGTCCTC	ACACAGTAGA	8040
	TGCAATCCAA	AAGAAGATCA	CAGAAACCAA	GCAGTTGGCC	AAAGACCTCC	GTCAACGGCA	8100
15	GATAAGTGTA	GACGTGGCAA	ATGATTTGGC	ACTGAAACTT	CTTCGGGACT	ATTCIGCTGA	8160
	TGATACCAGA	AAAGTACACA	TGATAACAGA	GAATATCAAT	ACTTCTTGGG	GAAACATTCA	8220
	TAAAAGAGTA	AGTGAGCAAG	AGGCTGCTTT	GGAAGAACT	CATAGATTAC	TGCAGCAGTT	8280
	CCCTCTGGAC	CTGGAGAAGT	TTCTTTCCTG	GATTACGGAA	GCAGAAACAA	CTGCCAATGT	8340
	CCTACAGGAC	GCTTCCCGTA	AGGAGAAGCT	CCTAGAAGAC	TCCAGGGGAG	TCAGAGAGCT	8400
20	GATGAAACCA	TGGCAAGATC	TCCAAGGAGA	AATTGAAACT	CACACAGATA	TCTATCACAA	8460
	TCTTGATGAA	AATGGCCAAA	AAATCCTGAG	ATCCCTGGAA	GGTTCGGATG	AAGCACCCCT	8520
	GTTACAAAGA	CGTTTGGATA	ACATGAATTT	CAAGTGGAGT	GAACITTCAGA	AAAAGTCTCT	8580
	CAACATTAGG	TCCCATTTGG	AAGCAAGTTC	TGACCAGTGG	AAGCGTTTGC	ATCTTCTCT	8640
	TCAGGAACCT	CTTGTTTGGC	TACAGCTGAA	AGATGATGAA	CTGAGCCGTC	AGGCACCCAT	8700
25	CGGTGGTGAT	TTCCAGCAG	TTCAGAAGCA	GAATGATATA	CATAGGGCCT	TCAAGAGGGA	8760
	ATTGAAAAC	AAAGAACCTG	TAATCATGAG	TACTCTGGAG	ACTGTGAGAA	TATTTCTGAC	8820
	AGAGCAGCCT	TTGGAAGGAC	TAGAGAACT	CTACCAGGAG	CCCAGAGAAC	TGCCTCCTGA	8880
	AGAAAGAGCT	CAGAATGTCA	CTCGGCTCCT	ACGAAAGCAG	GCTGAAGAGG	TCAACGCTGA	8940
	ATGGGACAAA	TTGAACCTGC	GCTCAGCTGA	TTGGCAGAGA	AAAATAGATG	AAGCTCTTGA	9000
30	AAGACTCCAG	GAACCTCAGG	AAGCTGCCGA	TGAACCTGGAC	CTCAAGTTGC	GCCAAGCTGA	9060
	GGTGATCAAG	GGATCCTGGC	AGCCAGTGGG	GGATCTCCTC	ATTGACTCTC	TGCAAGATCA	9120
	CCTTGAAAAA	GTCAAGGCAC	TTCGGGGAGA	AATTGCACCT	CTTAAAGAGA	ATGTCAATCG	9180
	TGTCAATGAC	CTTGACATC	AGCTGACCAC	ACTGGGCATT	CAGCTCTCAC	CTTATAACCT	9240
	CAGCACTTTG	GAAGATCTGA	ATACCAGATG	GAGGCTTCTA	CAGGTGGCTG	TGGAGGACCG	9300
35	TGTCAGACAG	CTGCATGAAG	CCCACAGGGA	CTTTGGTCTC	GCATCCAGC	ACTTCCCTTC	9360
	CACTTCAGTT	CAGGGTCCCT	GGGAGAGAGC	CATCTCACCA	AACAAAGTGC	CCTACTATAT	9420
	CAACCACGAG	ACCCAAACCA	CTTGTGGGGA	CCACCCAAA	ATGACAGAGC	TCTACCAGTC	9480
	TTTAGCTGAC	CTGAATAATG	TCAGGTTCTC	CGCGTATAGG	ACTGCCATGA	AGCTCAGAAG	9540
	GCTCCAGAAG	GCCCTTTGCT	TGGATCTCTT	GAGCCTGTCA	GCTGCATGTG	ATGCCCTGGA	9600
40	CCAGCACAAC	CTCAAGCAAA	ATGACCAGCC	CATGGATATC	CTGCAGATAA	TTAACTGTTT	9660
	GACTACAATT	TATGATCGTC	TGGAGCAAGA	GCACAACAAT	CTGGTCAATG	TCCCTCTCTG	9720
	TGTGGATATG	TGTCTCAACT	GGCTTCTCAA	TGTTTATGAT	ACGGGACGAA	CAGGGAGGAT	9780
	CCGTGTCCCTG	TCTTTTAAAA	CTGGCATCAT	TTCTCTGTGT	AAAGCACACT	TGGAAGACAA	9840
	GTACAGATAC	CTTTTCAAGC	AAGTGGCAAG	TTCAACTGGC	TTTTGTGACC	AGCGTAGGCT	9900
45	GGGTCTTCTT	CTGCATGATT	CTATTCAAAT	CCCAAGACAG	TTGGGTGAAG	TTGCTTCCTT	9960
	TGGGGGCGAGT	AACATTGAGC	CGAGTGTGAG	GAGCTGCTTC	CAATTTGCCA	ATAATAAACC	10020
	TGAGATTGAA	GCTGCTCTCT	TCCTTGACTG	GATGCGCCTG	GAACCCAGT	CTATGGTGTG	10080

	GCTGCCCGTC	TTGCACAGAG	TGGCTGCTGC	TGAAACTGCC	AAGCATCAAG	CCAAGTGTAA	10140
	CATCTGTAAG	GAGTGTCCAA	TCATTGGATT	CAGGTACAGA	AGCCTAAAGC	ATTTTAATTA	10200
	TGACATCTGC	CAAAGTTGCT	TTTTTCTGG	CCGAGTTGCA	AAGGGCCATA	AAATGCACTA	10260
	CCCCATGGTA	GAGTATTGCA	CTCCGACTAC	ATCCGGAGAA	GATGTTGCG	ACTTCGCCAA	10320
5	GGTACTAAAA	AACAAATTTT	GAACCAAAAG	GTATTTTTCG	AAGCATCCCC	GAATGGGCTA	10380
	CCTGCCAGTG	CAGACTGTGT	TAGAGGGGGA	CAACATGGAA	ACTCCCGTTA	CTCTGATCAA	10440
	CTTCTGGCCA	GTAGATTCTG	CGCCTGCCTC	GTCCCCCAG	CTTTCACACG	ATGATACTCA	10500
	TTACAGCATT	GAACATTATG	CTAGCAGGCT	AGCAGAAATG	GAAAACAGCA	ATGGATCTTA	10560
	TCTAAATGAT	AGCATCTCTC	CTAATGAGAG	CATAGATGAT	GAACATTTGT	TAATCCAGCA	10620
10	TTACTGCCAA	AGTTTGAACC	AGGACTCCCC	CCTGAGCCAG	CCTCGTAGTC	CTGCCCAGAT	10680
	CTTGATTTCC	TTAGAGAGTG	AGGAAAGAGG	GGAGCTAGAG	AGAATCCTAG	CAGATCTTGA	10740
	GGAAGAAAAC	AGGAATCTGC	AAGCAGAATA	TGATCGCCTG	AAGCAGCAGC	ATGAGCATAA	10800
	AGGCCTGTCT	CCACTGCCAT	CTCCTCCTGA	GATGATGCCC	ACCTCTCCTC	AGAGTCCCAG	10860
	GGATGCTGAG	CTCATTGCTG	AGGCTAAGCT	ACTGCGCCAA	CACAAAGGAC	GCCTGGAAGC	10920
15	CAGGATGCAA	ATCCTGGAAG	ACCACAATAA	ACAGCTGGAG	TCTCAGTTAC	ATAGACTGAG	10980
	ACAGCTCCTG	GAGCAGCCCC	AGGCTGAAGC	TAAGGTGAAT	GGCACCACGG	TGTCCTCTCC	11040
	TTCCACCTCT	CTGCAGAGGT	CAGATAGCAG	TCAGCCTATG	CTGCTCCGAG	TGGTTGGCAG	11100
	TCAAACCTCA	GAATCTATGG	GTGAGGAAGA	TCTTCTGAGT	CCTCCCCAGG	ACACAAGCAC	11160
	AGGGTTAGAA	GAAGTGATGG	AGCAACTCAA	CAACTCCTTC	CCTAGTTCAA	GAGGAAGAAA	11220
20	TGCCCCCGGA	AAGCCAATGA	GAGAGGACAC	AATGTAGGAA	GCCTTTTCCA	CATGGCAGAT	11280
	GATTTGGGCA	GAGCGATGGA	GTCCCTTAGTT	TCAGTCATGA	CAGATGAAGA	AGGAGCAGAA	11340
	TAAATGTTTT	ACAACCTCTG	ATTCCCGCAT	GGTTTTTATA	ATATTGCTAC	AACAAAGAGG	11400
	ATTAGACAGT	AAGAGTTTAC	AAGAAATAAA	ATCTATATTT	TTGTGAAGGG	TAGTGGTACT	11460
	ATACTGTAGA	TTTCAGTAGT	TTCTAAGTCT	GTTATTGTTT	TGTTAACAAT	GGCAGGTTTT	11520
25	ACACGTCTAT	GCAATTGTAC	AAAAAAGTTA	AAAGAAAACA	TGTAAAATCT	TGATAGCTAA	11580
	ATAACTTGCC	ATTTCTTTAT	ATGGAACGCA	TTTTGGGTTG	TTTAAAAATT	TATAACAGTT	11640
	ATAAAGAGAG	ATTGTAAACT	AAAGTGTGCT	TTATAAAAAA	AGTTGTTTAT	AAAAACCCCT	11700
	AAACAAACAC	ACACGCACAC	ACACACACAC	ACACACACAC	ACACACACAC	GCACACATAC	11760
	ATGCACGAAC	CCACCACACA	CACACACACA	CACACACACA	CTGAGGCAGC	ACATTGTTTT	11820
30	GCATTACTTT	AGCGTGGTAT	TCATATGGAA	TTCATGACGT	TTTTTTATTT	TCTTGCATAC	11880
	GAACCCCAAC	AAATGACTGC	TTCATATTGC	TCTTTTGAGA	ATTGTTGACT	GAGTGGGGCT	11940
	GGCTATGGGC	TTTCATTTTA	TACATCTATA	TGTCTACAAG	TATATAAATA	CTATAGGTAT	12000
	ATAGATAAAT	AGATATGAAG	TTACTTCTTC	AAATGTTCTT	GCCACTTCCT	AATGGAAATT	12060
	GCTTCTAGTC	ATCTGGGCTT	ATCTGCTTGG	GCAAGAGTGA	ATTTTCCCTG	GAGCCCAAAG	12120
35	CCAGGAGACT	ACCGCCACAC	TAAAATATTG	TCTAGGGCTC	CAGATGTTTC	TAGTTTTAAA	12180
	CTTTCCACTG	AGAGCTAGAG	GATTCATTTT	TTTCAAGGAA	CATGCCAATG	AATACACAGG	12240
	ACTTACTATC	ATAGTAATTT	GTTGGCTGAT	ATATTCAACT	TCTTACTGTT	GGGTTATATT	12300
	TAATGATGTT	TCTGCAATAG	AACATCAGAT	GACATTTTTA	ACTCCCAGAC	AGTAGGAGGA	12360
	AGATGGTAGG	AGCTAAAGGT	TGCGGCTCCT	CAGTCAATTT	ATATGAGGGG	AGCAACAAC	12420
40	CTGTAAAAGA	ATGGATGAAT	ATTTACAAC	ATACATATAA	ACATCTCTAT	AATTACAAC	12480
	AAATGTTTCT	GCCCTCTTCA	TAAACTCAAC	CTGAAGTGGG	TGGTTTTGTT	GTTGTTGTTG	12540
	TGTTGTTGTT	TGATGATGAT	GATGAATTTT	AGATTTTAGA	TTTTTTGGGT	TTTTTTTTCT	12600
	TCATTGTGAT	GATTTTTTTT	TTTAATGCTG	CAAGACTTAG	GATTACTGTT	AAGAAAGTAA	12660
	CCCAATCACA	TTGTGACCCT	GGTGAATATC	AGTCCAGAAG	CCCATGAAC	GCAATTTGCT	12720
45	CCTTTGCAAT	GGTTTCCCTG	CAAGTAACTC	CACACAGGAT	TGTGGGTGAG	AAGGCACAGT	12780
	GGTTGGAAAG	TTTTGAGAGC	AAAAGCGTCT	CCAAACCTC	TGGTCTAGTT	GACGGGCTGA	12840
	AATGTCTAAA	CAAATGCAAG	TCATTGAACC	AGGAGAAAAA	GTGCAACAGA	AAGCTAAGGA	12900

SUBSTITUTE SHEET (RULE 26)

CTGCTAGGAA GAGCTTTACT CCTCTCATGC CAGTTTCTTC TTCTTAGCAT TTAAAGAGCA 12960
 TTCTCTCAAT AGAAATCACT GTCCTATCAT TTTGCAAATC TGTTACCTCT AACGTCAAGT 13020
 GTAATTAAC TCTAGCGAGT GGGTTTTGTC CATTATTAAT TGTAATTAAC ATCAAACACA 13080
 GCTTCTCATG CTATTTCTAC CTCACHTTGG TTTTGGGGTG TTTCTAGTAA TTGTGCACAC 13140
 5 CTAATTTTAC AACTTCACCA CTTGTCTGTT GTGTGGACAC CAGTTTCTTT TTTTCATTTA 13200
 TAATTTCCAA AAGAAAACCC AAAGCTCTAA GATAACAAAT TGAAATTTGG TTCTGGTCTT 13260
 GCTTTTCTCT CTCCTCTCTCC TTTATGTGGC ACTGGGCATT TTCTTTATCC AAGGATTTGT 13320
 TTTACCAAG ATTTAAAACA AGGGGTTTCT TTCCTACTAA GAAGTTTTAA GTTTCATTCT 13380
 AAAATCCAAG GTAGATAGAG TGCATAGTTT TGTTTTAATC TTTTCGTTTT ATCTTTTAGA 13440
 10 TATTAGTTCT GGAGTGAATC TATCAAAATA TTTGAATAAA AACTGAGAGC TTTATTGCTG 13500
 ATTTTAAGCA TAATTTGGAC ATCATTTTCT GTTCTTTATA ACCATCAAGT ATTAAAGTGT 13560
 AAATCATAAT CAGTGTAAC GAAGCATAAT CATCACATGG CATGTATCAT CATTGTCTCC 13620
 AGGTACTGGA CTCTTACTTG AGTATCATAA TAGATTGTGT TTTAACACCA AACTGTAAAC 13680
 ATTTACTAAT TATTTTTTTA AACTTCAGTT TTAGTGATT TTTCAACAT ATCAGATTTT 13740
 15 ACCAAATATA TGCCTTACTA TTGTATTATA TTAGTGCTTT ACTGTGTATC TCAATAAAGC 13800
 ACGCAGTTAT GTTAC 13815

(2) INFORMATION FOR SEQ ID NO:18:

- 20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATACCGAGGC TGCAGTGTAC A

21

30 (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 50 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 35 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCAACCGAAC ATATCGCCAC GGCAGCCACC AACGATGCTG ATTGCCGTTT

50

40

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 57 base pairs
 45 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGTGGCTGCC GTGGCGATAT GTTCGGTTGC TAACTTTACC CTTCATCACT AAAGGCC 57

5 (2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

15 AAACGTACAG CGCCATGTTT ACCAG 25

SUBSTITUTE SHEET (RULE 26)

CLAIMS

1. In a method for gene therapy, the improvement which comprises using a bacteriophage containing genetic material to introduce a therapeutic polynucleotide into a target cell of a mammalian recipient, wherein the mammalian recipient is diagnosed as having a condition that is treatable by administration to the recipient of the therapeutic polynucleotide or a product thereof, the method comprising the steps of:

(1) contacting the bacteriophage with the target cell under conditions to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and to allow the bacteriophage to enter the target cell; and

(2) allowing the target cell to live under conditions such that the therapeutic polynucleotide is transcribed therein,

wherein the bacteriophage is incapable of injecting said genetic material into the target cell of the mammalian recipient.

2. The method of claim 1, wherein the bacteriophage propagates in a prokaryotic cell.

3. The method of claim 1, wherein the bacteriophage includes exogenous genetic material that is transcribed and translated in the target cell.

4. The method of claim 1, wherein the bacteriophage is selected from the group consisting of a lambda phage, a P1 phage, a P22 phage, a T1 phage, a T2 phage, a T3 phage, a T4 phage, a T5 phage, a T6 phage, a T7 phage, a P2 phage, a P4 phage, an Mu phage, a PM2 phage, an N4 phage, an SPO1 phage, a PBS1 phage, and a PBS2 phage.

5. The method of claim 1, wherein the therapeutic polynucleotide is operably coupled to a promoter.

6. The method of claim 1, wherein contacting the bacteriophage with the target cell is performed in vitro.

7. The method of claim 1, wherein the bacteriophage contains exogenous genetic material that contains a cell-specific promoter that effects transcription and translation of the therapeutic polynucleotide in the target cell.

8. The method of claim 1, wherein the bacteriophage contains one or more modified tail proteins, wherein the modified tail proteins cannot facilitate injection of the genetic material into the target cell of the mammalian recipient.

9. A method for introducing an exogenous polynucleotide into a mammalian cell comprising:

(1) contacting a bacteriophage containing genetic material with the target cell under conditions to permit selective binding of a ligand on the surface of the bacteriophage to a receptor on the surface of the target cell and to allow the bacteriophage to enter the target cell; and

(2) allowing the target cell to live under conditions such that the therapeutic polynucleotide is transcribed therein,

wherein the bacteriophage is incapable of injecting the genetic material into the target cell.

10. The method of claim 9, wherein the bacteriophage includes exogenous genetic material that is transcribed and translated in the target cell.

11. The method of claim 9, wherein the bacteriophage is derived from a bacteriophage selected from the group consisting of a lambda phage, a P1 phage, a P22 phage, a T1 phage, a T2 phage, a T3 phage, a T4 phage, a T5 phage, a T6 phage, a T7 phage, a P2 phage, a P4 phage, an Mu phage, a PM2 phage, an N4 phage, an SPO1 phage, a PBS1 phage, and a PBS2 phage.

12. The method of claim 9, wherein the exogenous polynucleotide comprises a therapeutic polynucleotide.

13. The method of claim 9, wherein the bacteriophage contains one or more modified tail proteins, wherein the modified tail proteins cannot facilitate injection of the genetic material into the target cell of the mammalian recipient.

14. The method of claim 9, wherein contacting the bacteriophage with the target cell is performed in vitro.

15. The method of claim 9, wherein the bacteriophage has a genome that contains a cell-specific promoter that effects transcription and translation of the therapeutic polynucleotide in the target cell.

16. A bacteriophage comprising:

(a) a bacteriophage genome containing an exogenous polynucleotide that can be transcribed in a mammalian cell; and

(b) a ligand contained on the surface of the bacteriophage, wherein the ligand

selectively binds to a receptor expressed on the surface of a mammalian cell,

wherein the bacteriophage is incapable of injecting the bacteriophage genome into the mammalian cell.

17. The composition of claim 16, wherein the composition is contained in an implant that is suitable for implantation into a mammalian recipient

18. A bacteriophage comprising:

a bacteriophage containing a bacteriophage genome that can be transcribed in a mammalian cell, wherein the bacteriophage contains avidin on its surface and wherein the bacteriophage is incapable of injecting the bacteriophage genome into the mammalian cell.

19. The bacteriophage of claim 18, wherein the bacteriophage is contained in a kit with instructions for attaching a biotinylated ligand to the bacteriophage to form a ligand-labeled bacteriophage.

20. A kit comprising a container including:

(1) a first container containing a bacteriophage having a surface and a genome, wherein the bacteriophage is incapable of injecting the bacteriophage genome into a mammalian cell;

(2) a second container containing an agent for attaching a ligand to the surface of a bacteriophage, wherein the ligand binds to a receptor on the surface of the mammalian cell; and

(3) instructions for attaching the agent to the surface of the bacteriophage.

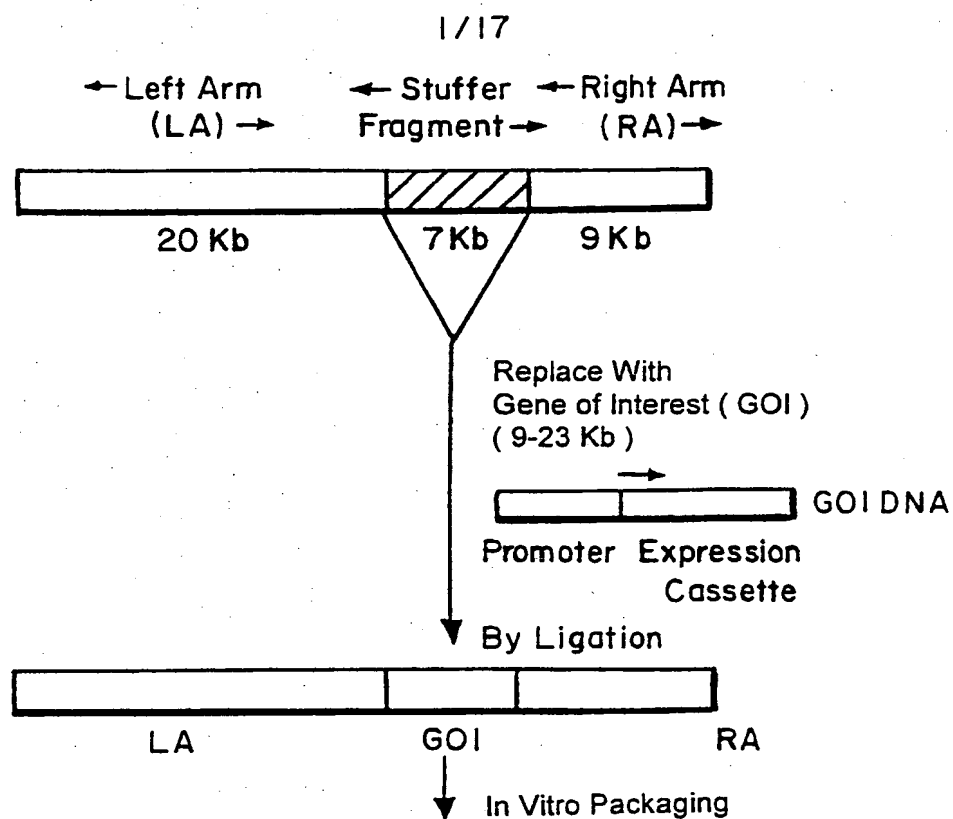


FIG. 1A

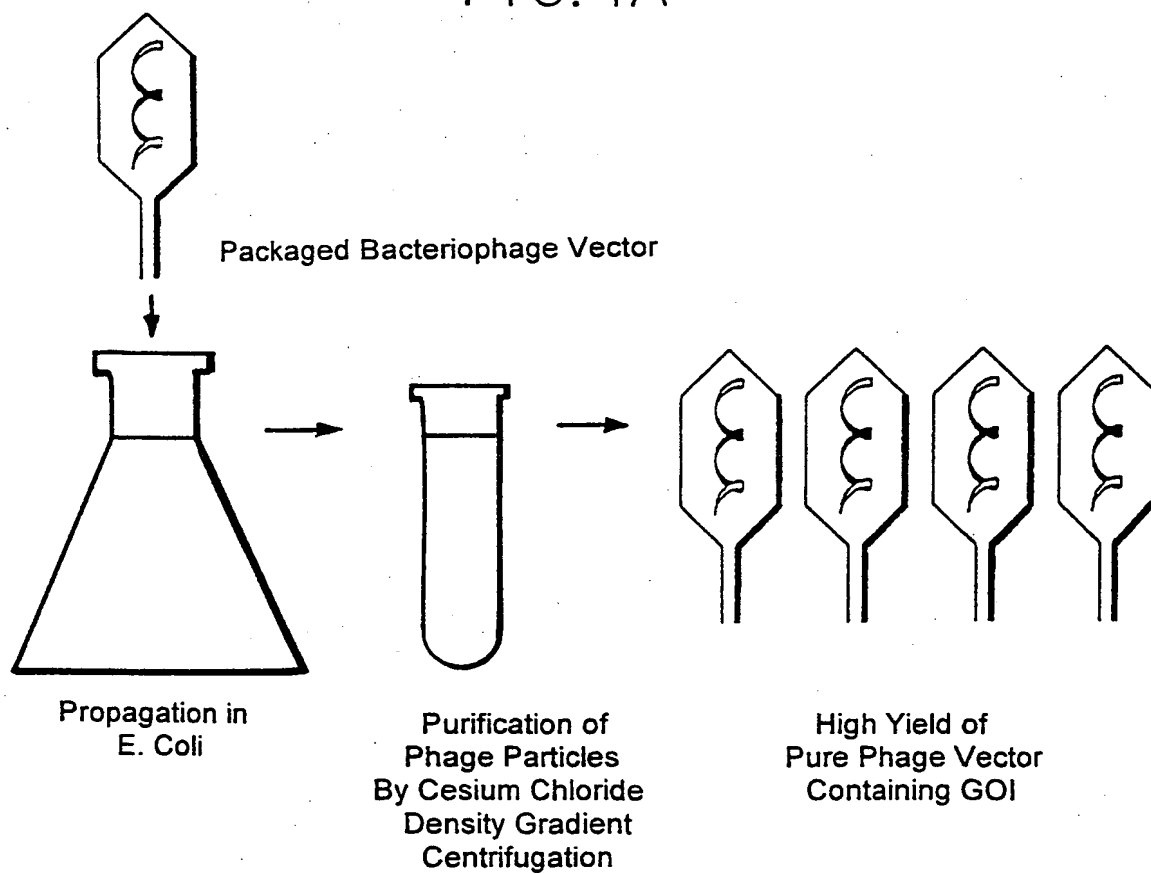


FIG. 1B

SUBSTITUTE SHEET (RULE 26)

2 / 17

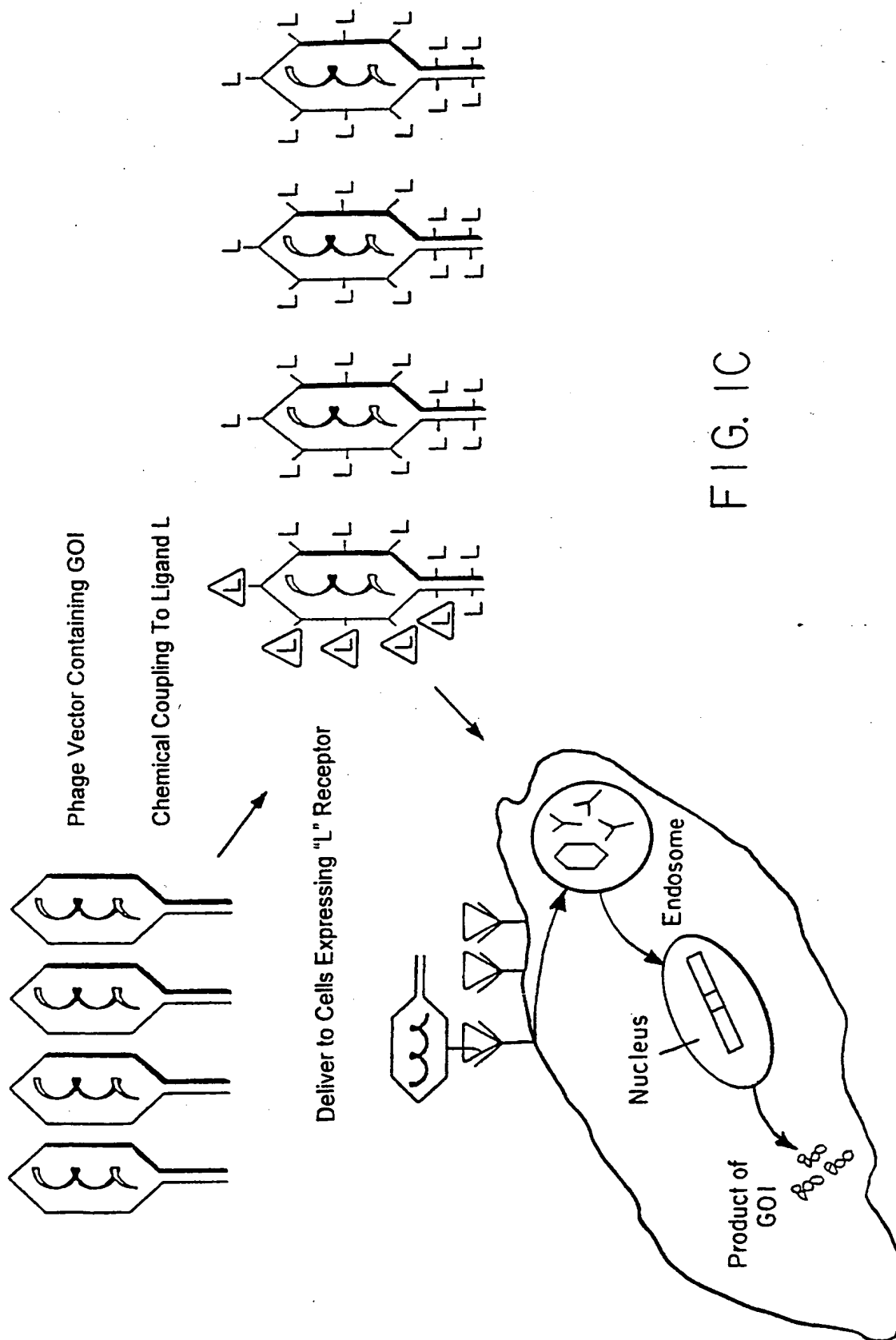


FIG. 1C

3/17

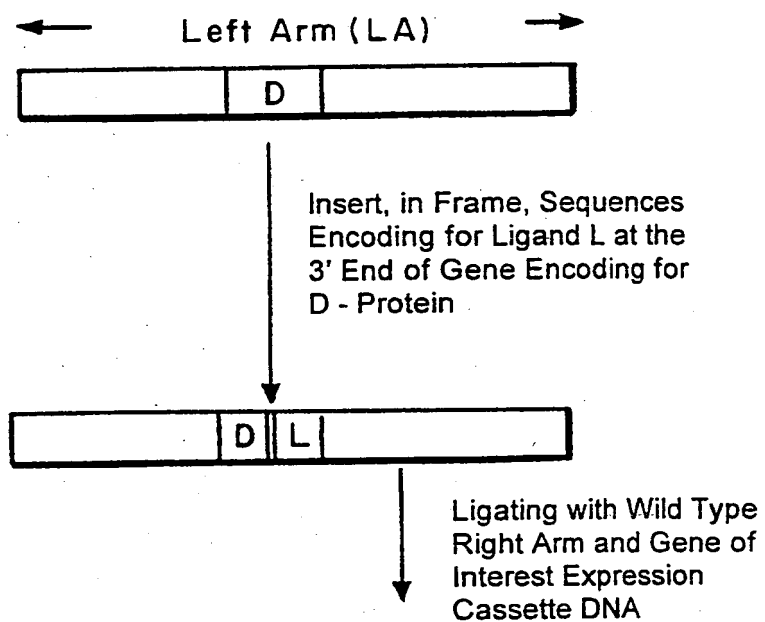


FIG. 2

4/17

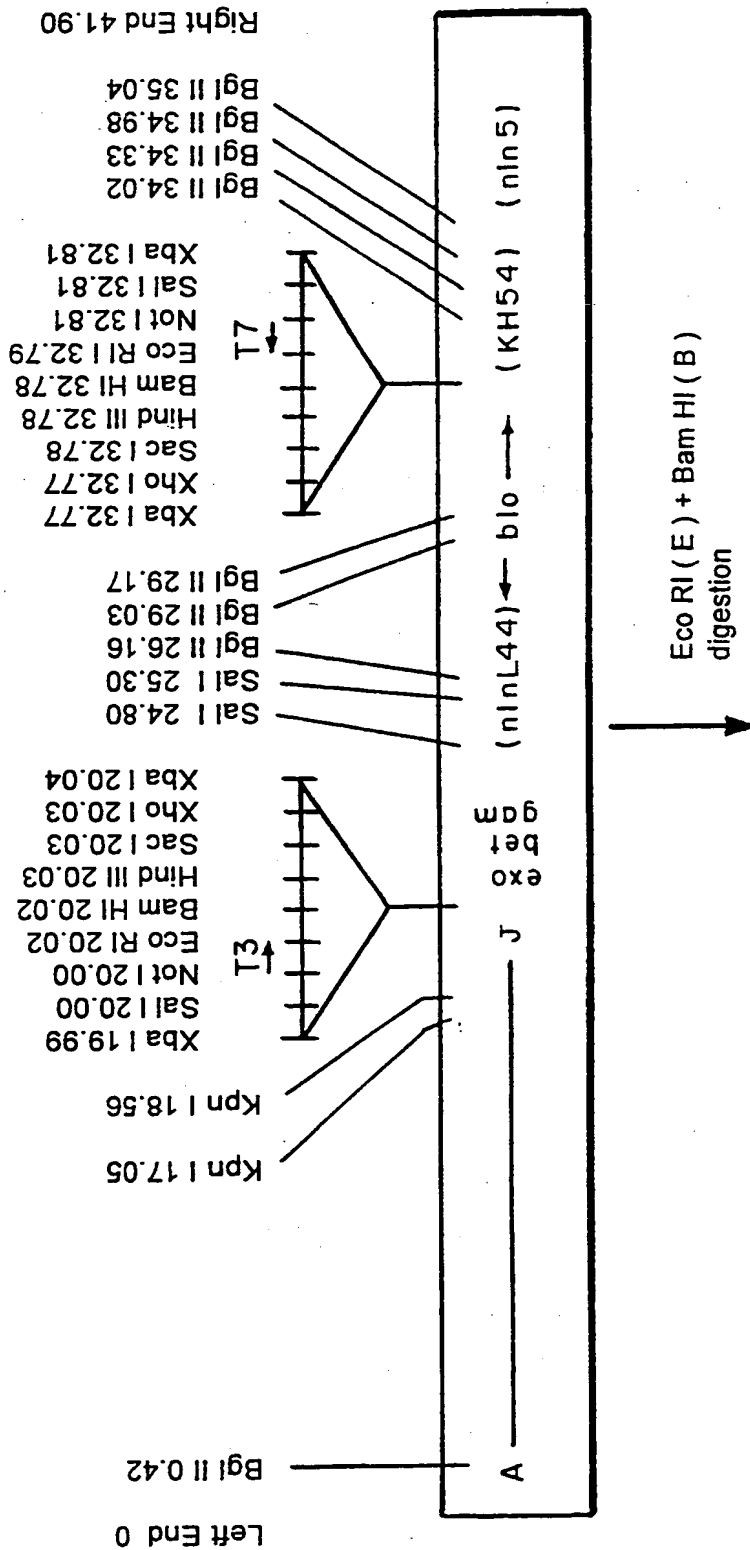


FIG. 3A

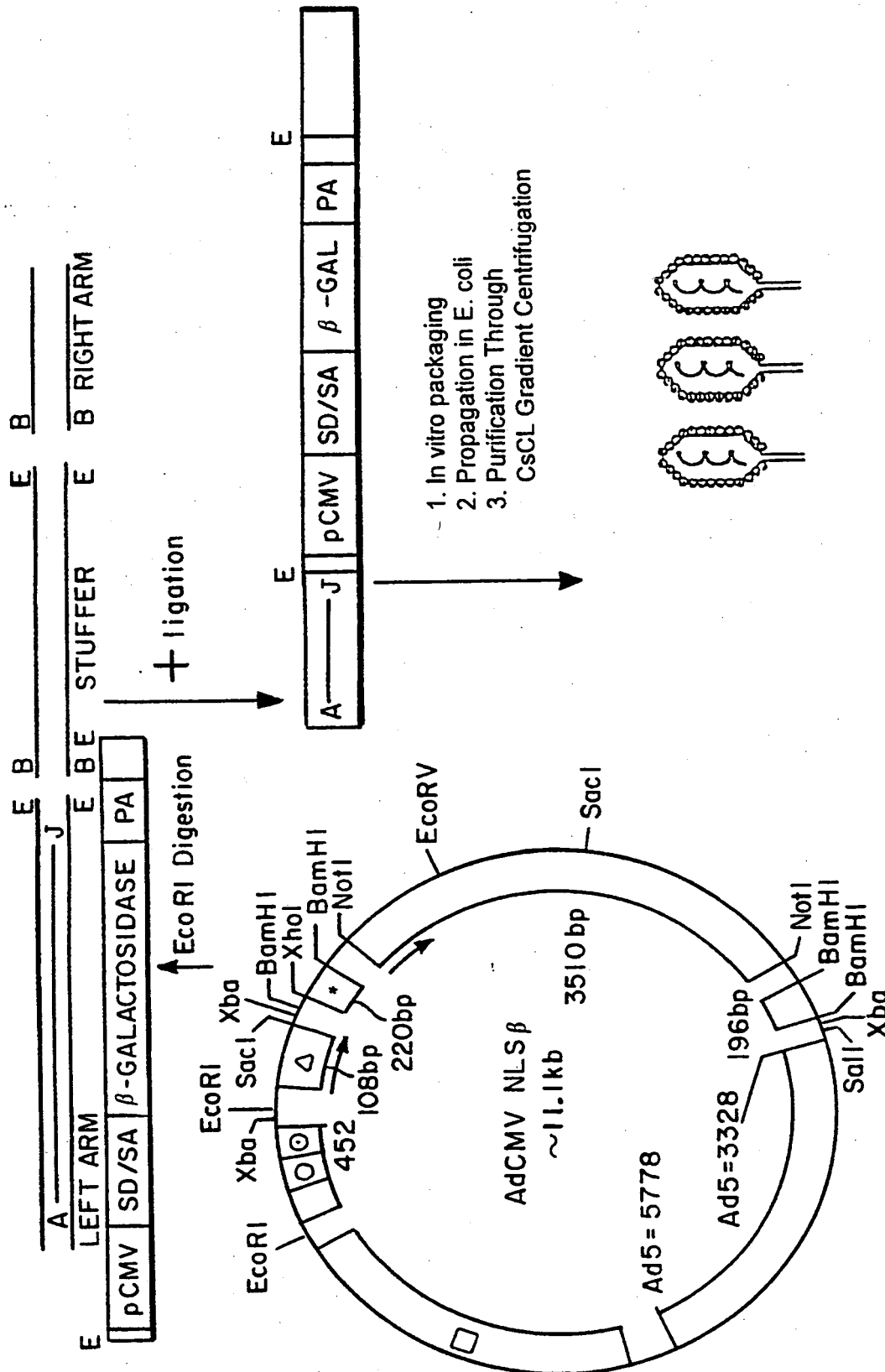


FIG. 3B

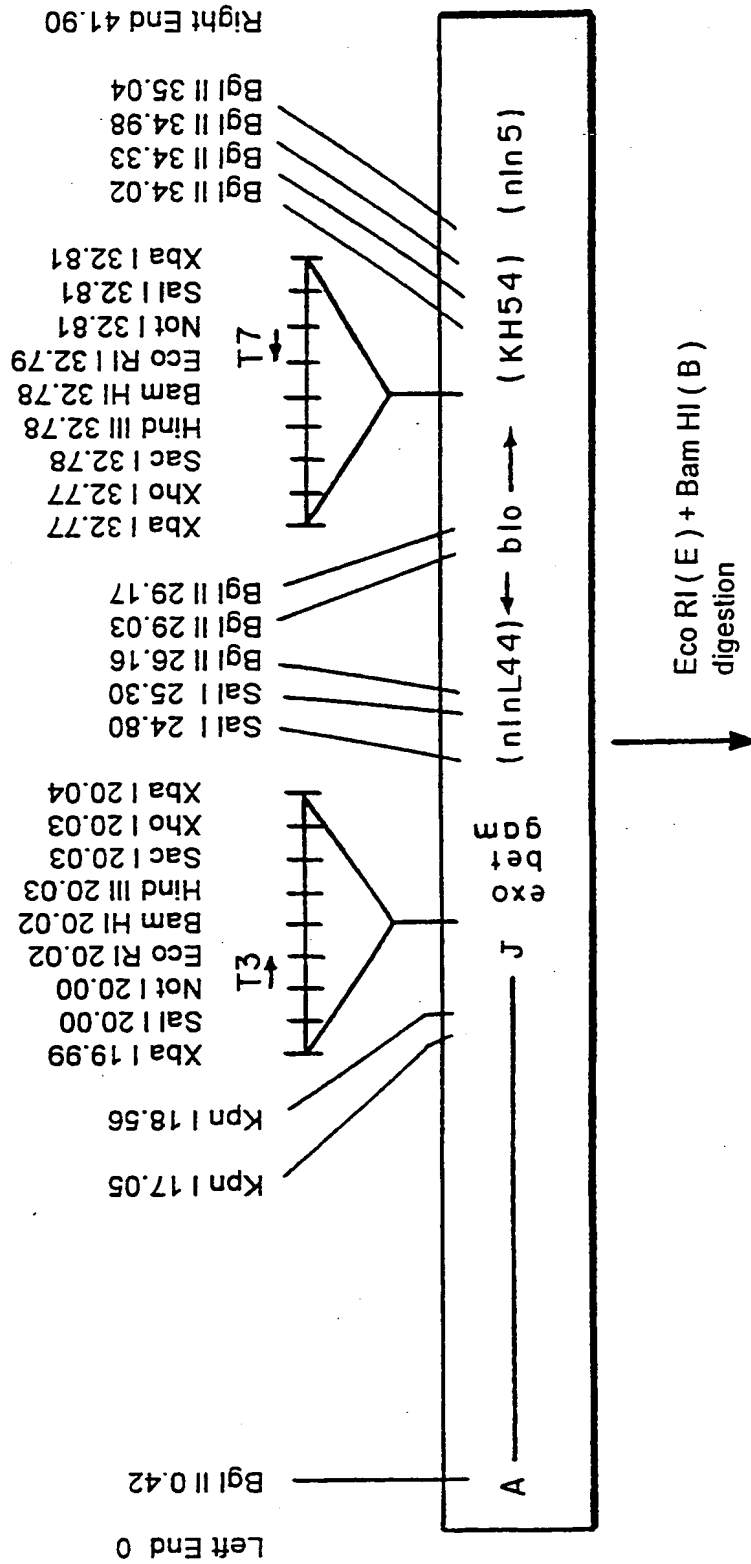


FIG. 4A

7/17

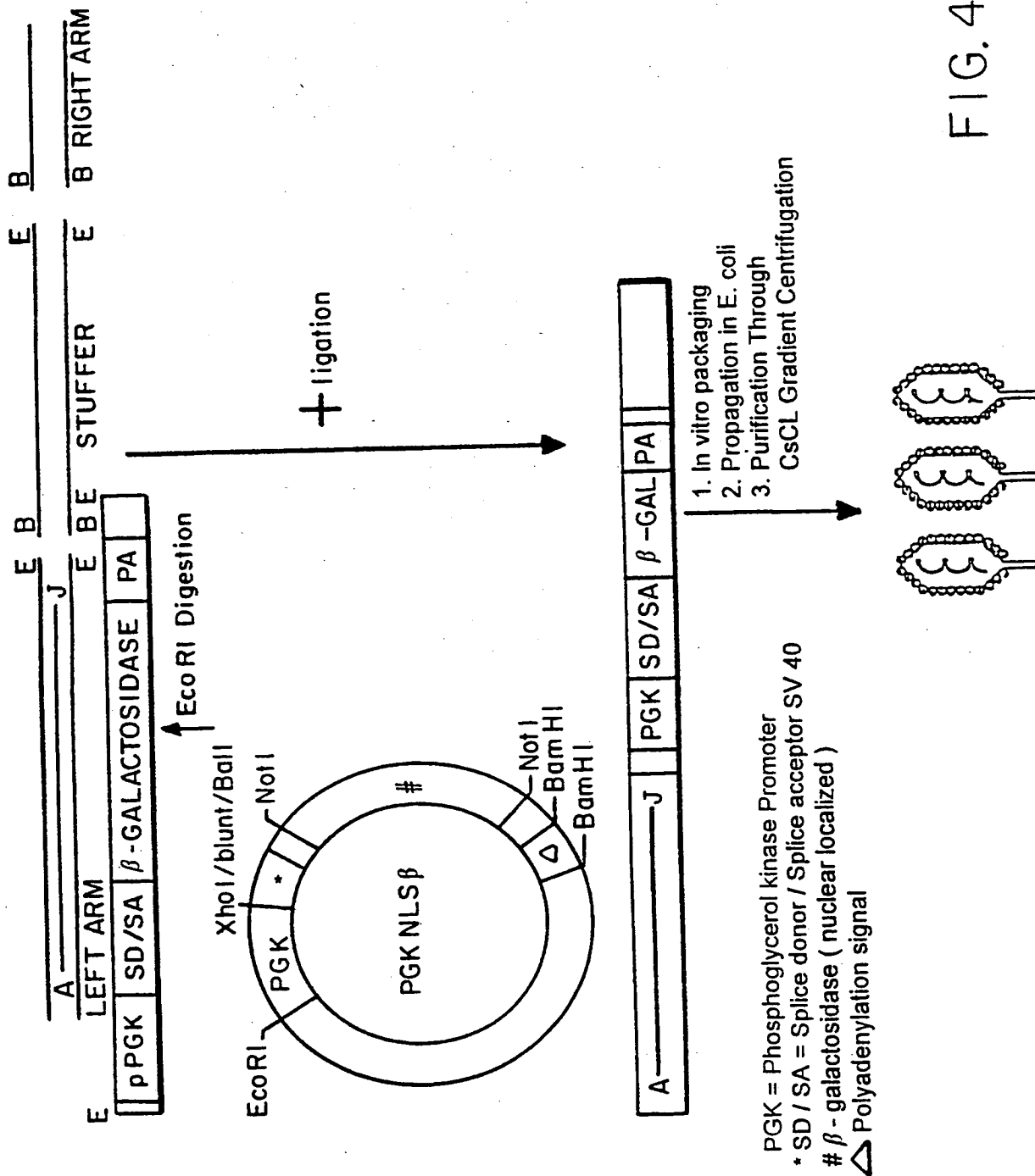


FIG. 4B

8 / 17

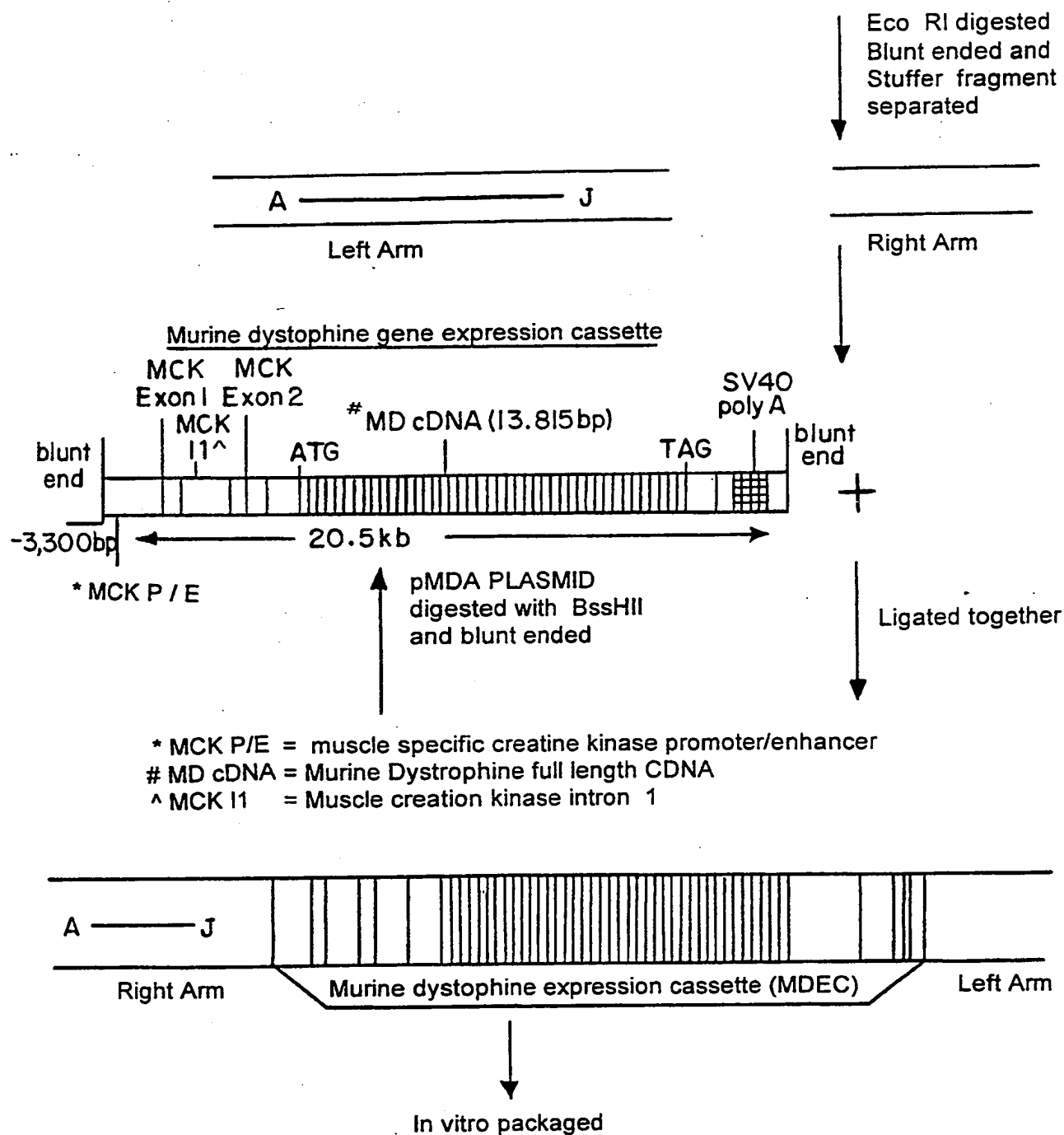
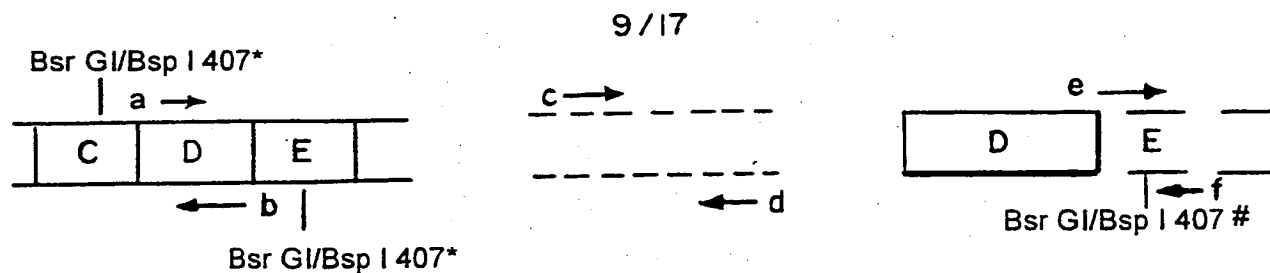


FIG.5

Wt λ genomic DNA template

Heregulin cDNA template

Wt λ genomic DNA template

* the location of this site is position 5220

the location of this site is position 6140

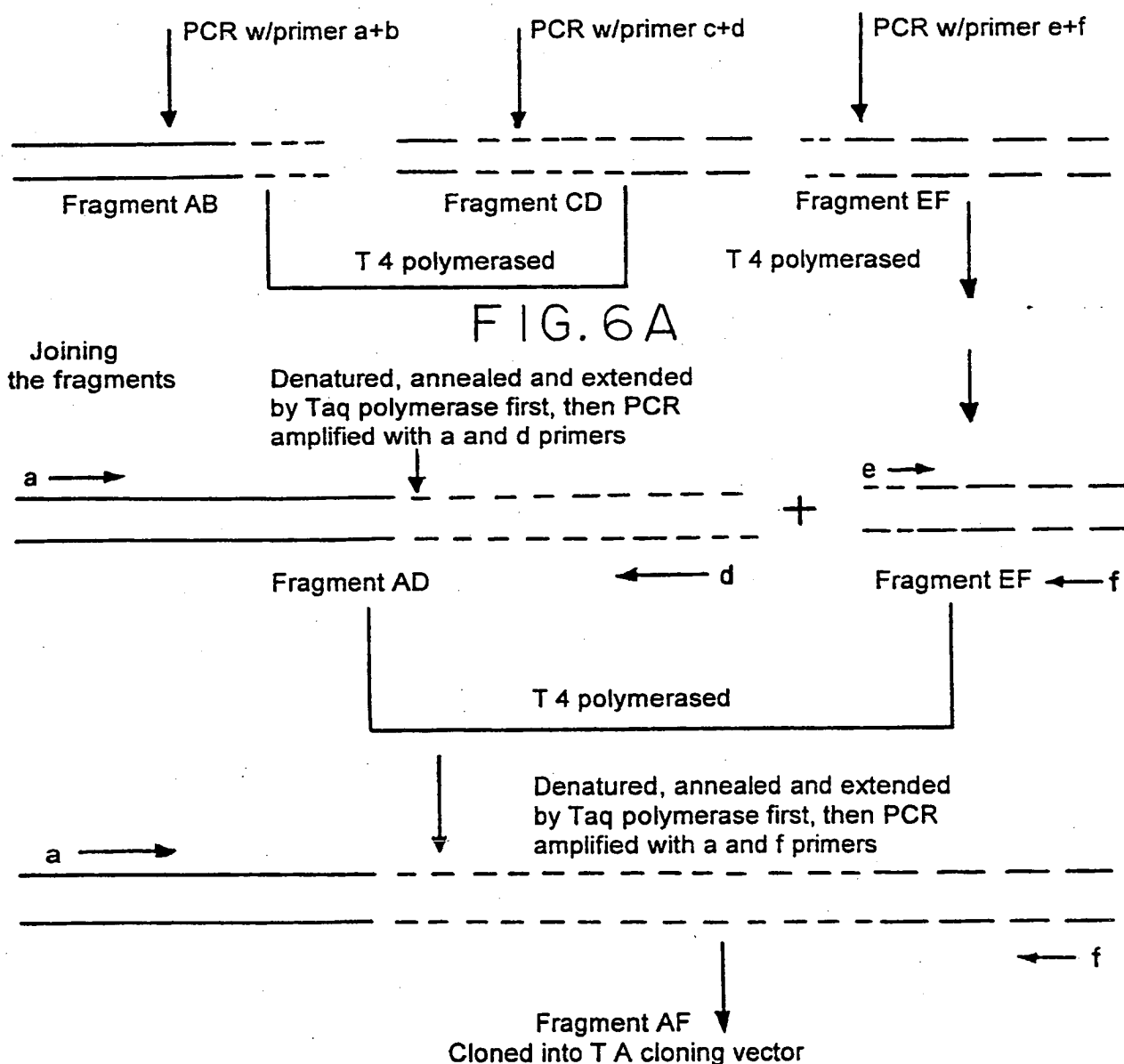


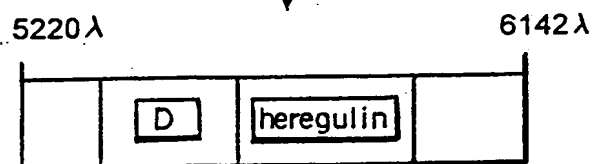
FIG. 6B

SUBSTITUTE SHEET (RULE 26)

10/17

T A plasmid containing fragment AF

digested with Bsr GI
and gel purified

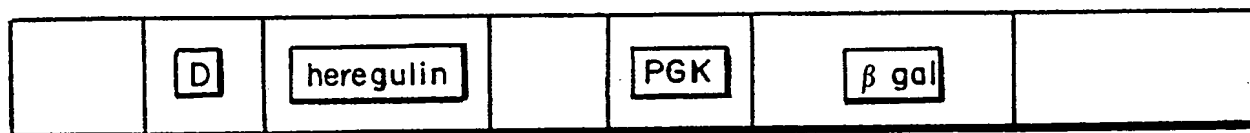


ligated to Bsr GI digested
gel purified fragments of PGK/
β - gal/ λ DASH II DNA

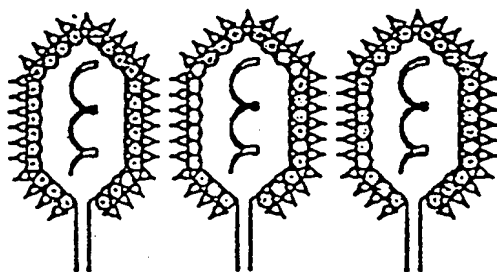
Fragment 1: *1-5220
Fragment 2: *5221-D- here - *6142
Fragment 3: *6143- 15855*

Fragment 4: *15856-PGK- β gal- λ *41900

* λ coordinates



Package with in vitro packaging extract



λ phage particle bearing a chimeric
D- ligand (L, heregulin) protein (D L)
on its capsid

FIG. 6C

Generation of targeted bacteriophage vectors by fusing a cyc
RGD ligand onto the bacteriophage λ virion head specific D - gene product

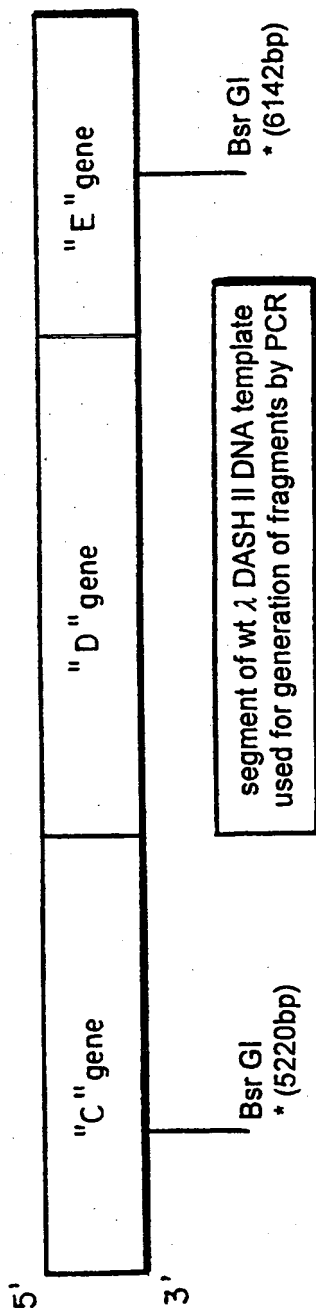


FIG. 7A

Using specially designed primers (as shown below), the cyc RGD coding sequences were fused onto the 3' end of the λ DASH II "D" gene as follows:

Generation of fragments for gene fusion

description of primers

"a" contains only wt sequence of the λ DASH I "C" GENE.

"b" has a 3' end that is complementary to 3' end of the wt "D" gene and a 5' end which contains the coding sequence to cyclic RGD.

"c" has a 5' end that is complementary to the 5' end of cyclic RGD and a 3' end which is complementary to the 5' end of the wt "E" gene.

"d" contains only wt sequence of the λ DASH II "E" gene

PCR generation and amplification of fragments

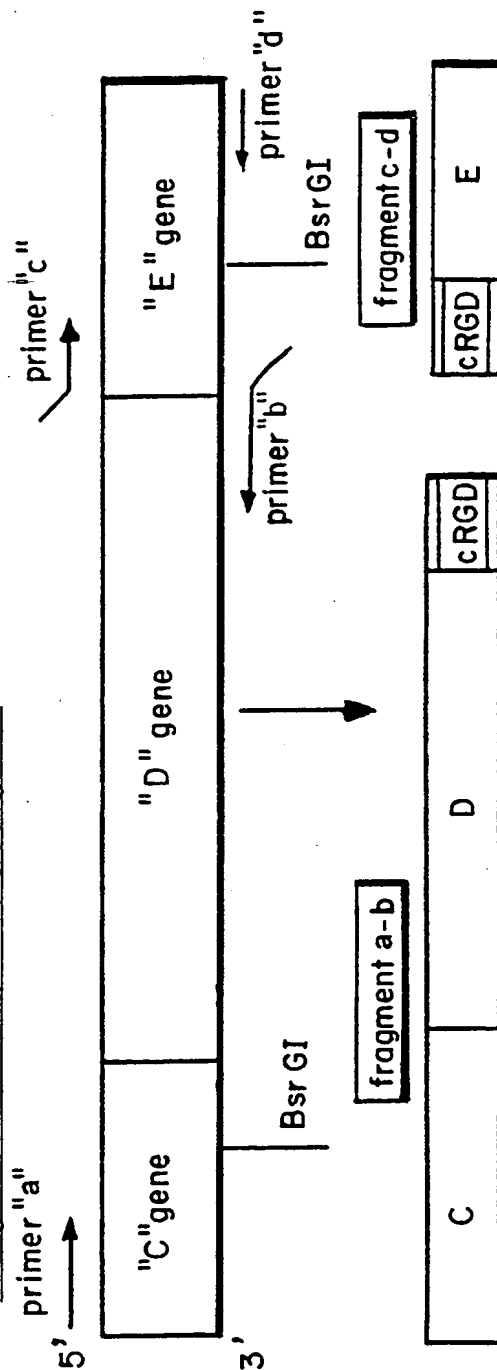


FIG. 7B

13/17

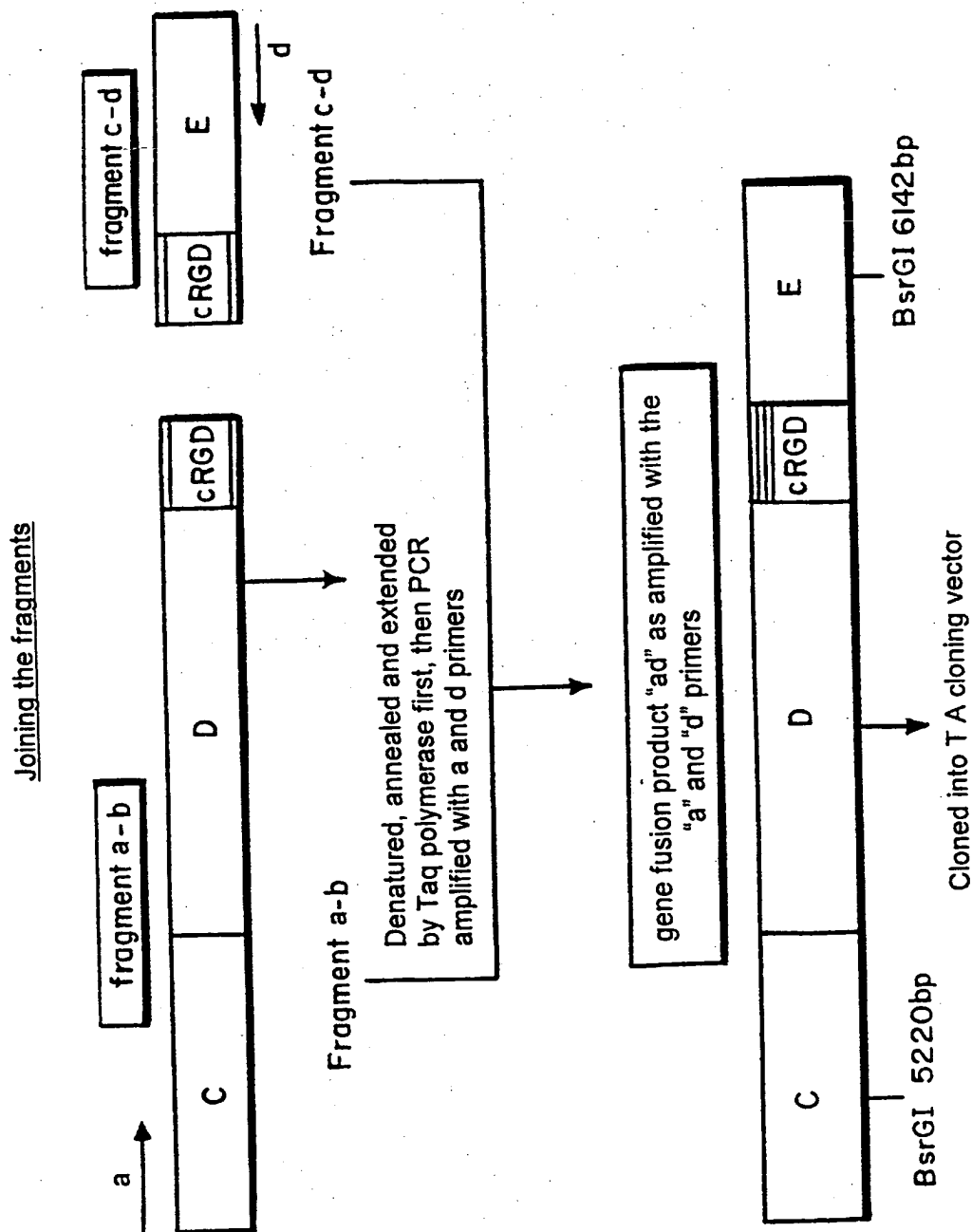


FIG. 7C

14 / 17

Generation of targeted λ phage vector expressing the cyc RGD-D chimeric capsid and containing the CMV β Gal reporter gene

preparation of λ DASH II / CMV β Gal vector

First, the λ DASH II / CMV β Gal bacteriophage vector was digested with Bsr GI and Eco RI* and cleaved into fragments. Fragment #2 (5220 - 6142bp) was then separated and removed by gel electrophoresis. (see below)

*co-digestion with Eco RI favors recombination of productive phage genome

Fragment 1: *1 - 5220

Fragment 2: *5221 - *6142

Fragment 3: *6143 - 15855*

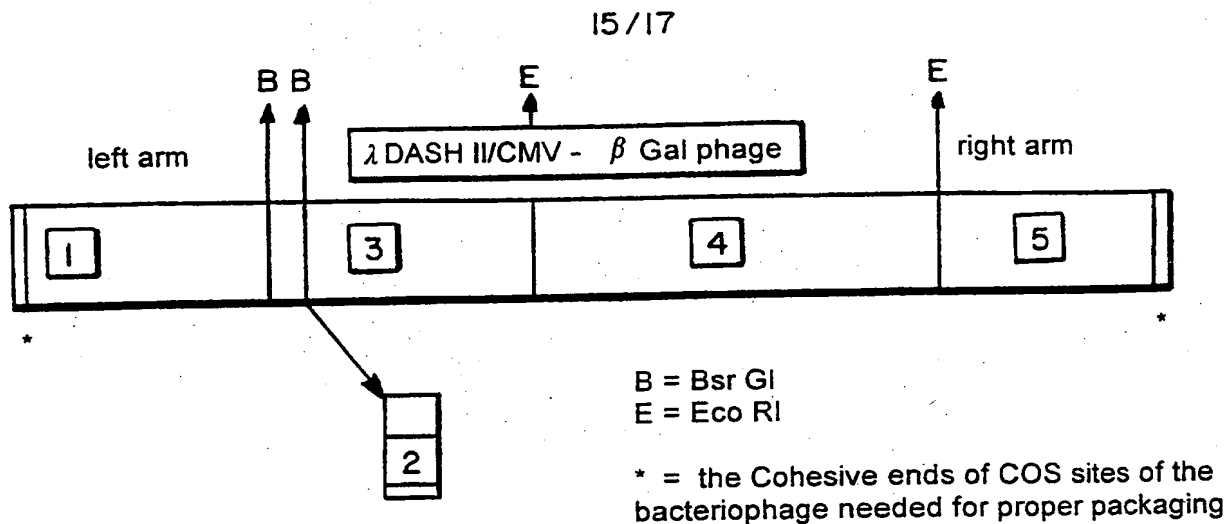
Fragment 4: *15856 - CMV - β gal - *41900

Fragment 5: *41900 - 48000

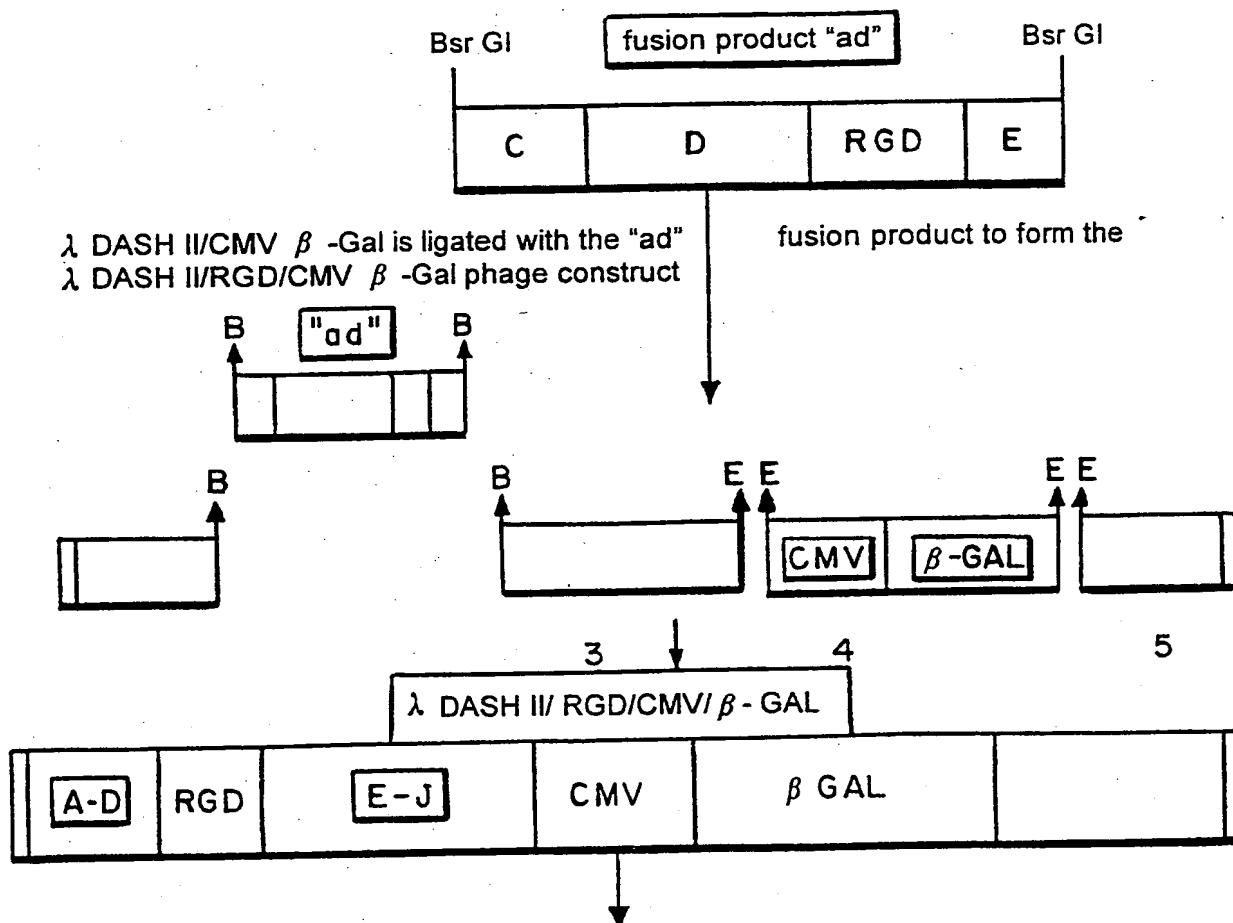
* λ coordinates



FIG. 7D-I



Next, the TA plasmid containing the "ad" gene fusion fragment is digested with Bsr GI



The ligated product was then packaged *in vitro* with *in vitro* packaging extract, the recombinant λ clones were characterized. Then λ DASHII/cyc RGD modified phage was then amplified and purified through CsCl banding

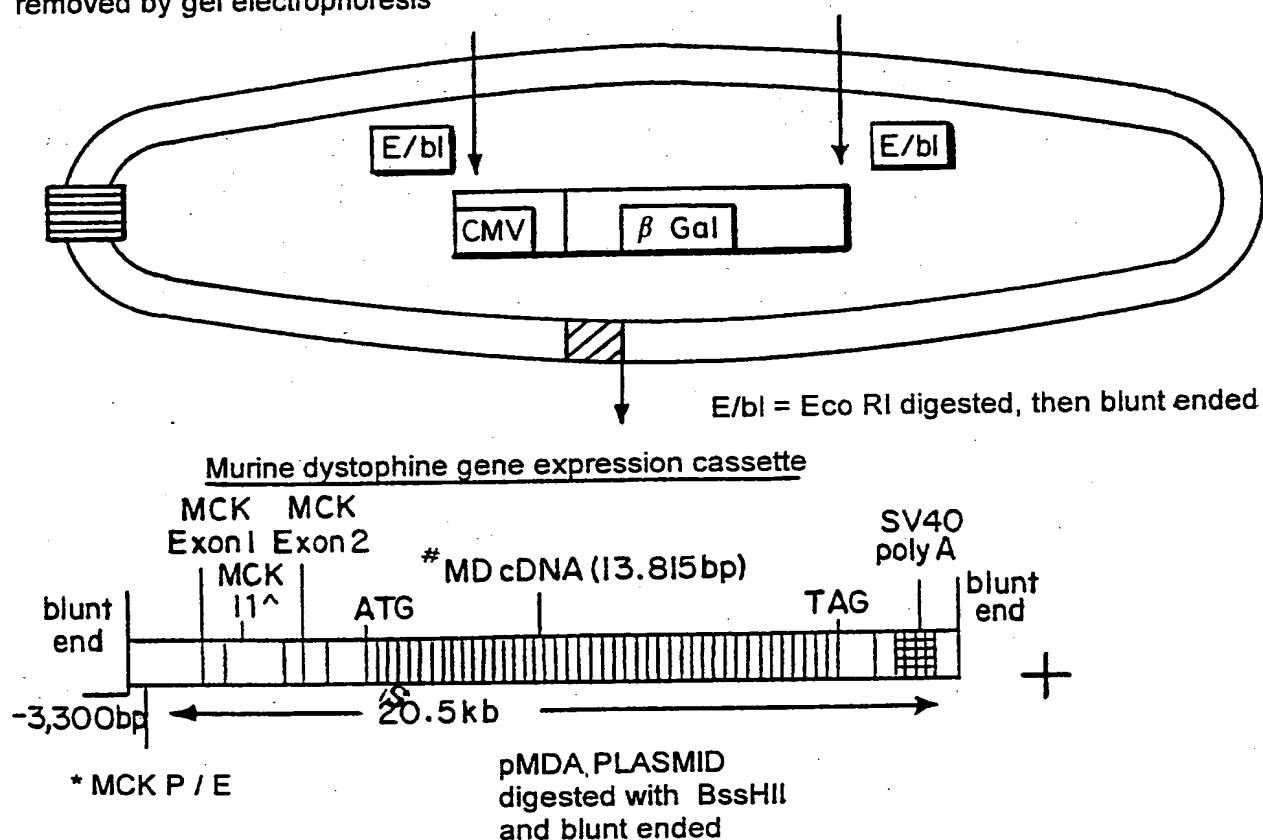
FIG. 7D-2
SUBSTITUTE SHEET (RULE 26)

16/17

This parental λ DASH II/cyc RGD/CMV β Gal modified bacteriophage was subsequently used as a vector for targeted gene transfer of other DNA constructs as described below

Generation of cyc RGD modified λ DASH II bacteriophage containing the murine dystrophin gene expression cassette

First, we ligated the COS sites of the cyc RGD modified λ DASH II bacteriophage DNA together to protect them during the blunt ending process
Then the phage was digested with Eco RI and the CMV β Gal expression cassette was removed by gel electrophoresis



- * MCK P/E = muscle specific creatine kinase promoter/enhancer
- # MD cDNA = Murine Dystrophin full length CDNA
- ^ MCK I1 = Muscle creatine kinase intron 1

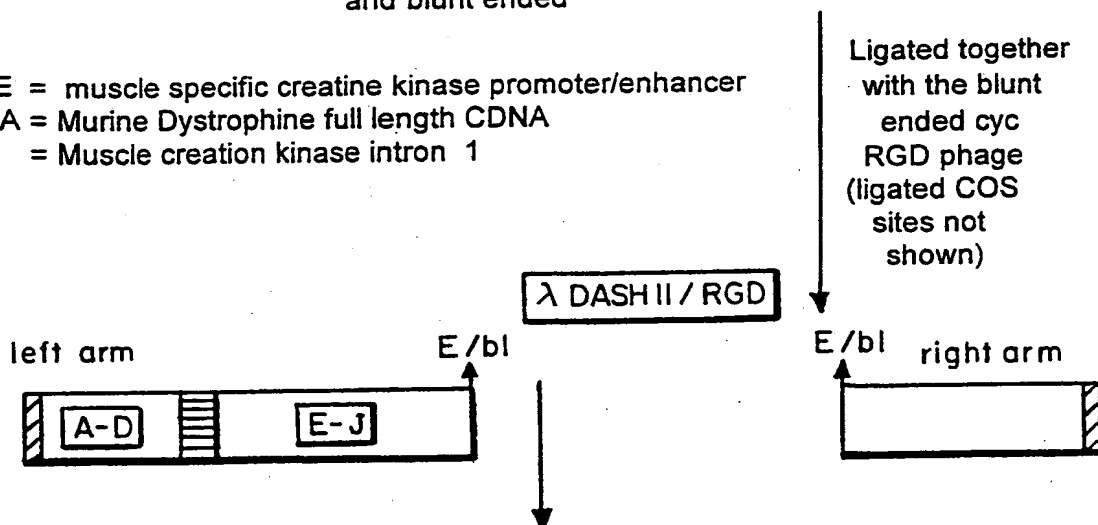


FIG. 7E

SUBSTITUTE SHEET (RULE 26)

17/17

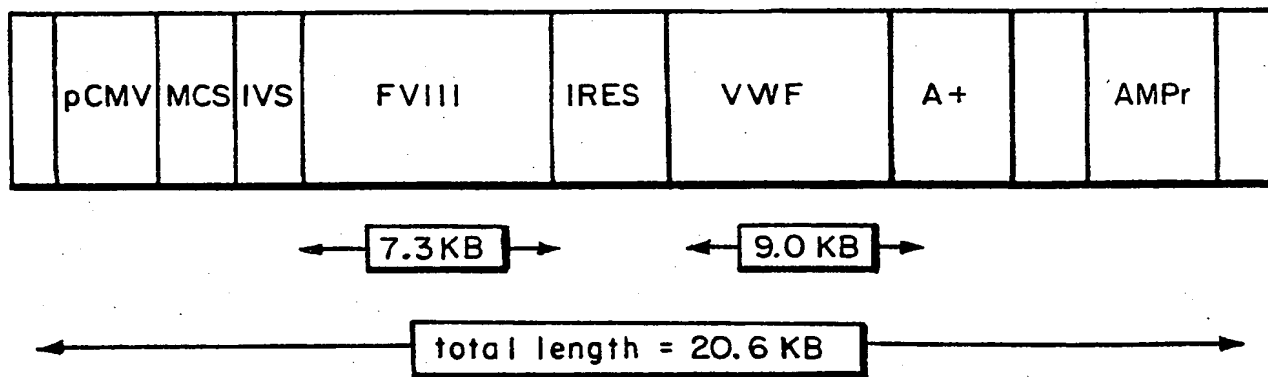


In vitro packaged, characterized, amplified and purified

Generation of cyc RGD modified λ DASH II bacteriophage containing the Factor VIII / IRES / Von Willebrand's Factor gene expression cassette

Construction of the plasmid containing the bicystronic expression cassette carrying the FVIII and the VWF genes

Gel purified, Eco RI digested, blunt ended, full length VWF cDNA was cloned into the Sma I site of the pIRES Δ neo (a derivative of the pIRESneo vector, in which the NEO gene was deleted by digestion with Kas I and Xba I and subsequent gel purification to remove the Kas I / Xba I fragment. The vector was then blunt ended and religated to generate the pIRES Δ neo vector (the parental pIRESneo was obtained from Clontech, CA.) The resulting plasmid, pIRES Δ neo / VWF, was then digested with Eco RI, blunt ended, and ligated to Xho / Sal digested, blunt ended and gel purified full length factor VIII cDNA fragment. The resulting plasmid (as shown below) constitutes the bicystronic expression cassette containing the FVIII / VWF genes.



Generation of the cyc RGD / λ DASH II / FVIII / IRES / VWF bacteriophage

Nru I digested pIRES Δ neo / FVIII / IRES / VWF plasmid DNA was used for ligation with the blunted left and right arms of the cyc RGD / λ DASH II prepared DNA. The ligated product was then *in vitro* packaged and clones were characterized, amplified and purified by CsCl banding.

FIG. 7F

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12928**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 35/76; C12N 7/00, 7/01, 15/09, 15/85, 15/86

US CL :424/93.1, 93.6; 435/172.1, 172.3, 235.1, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.6; 435/172.1, 172.3, 235.1, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS

search terms: bacteriophage#, phage#, eukaryotic, eucaryotic, mammal###, human, mouse, transfect###, transduc####, gene therapy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/21007 (CHIRON VIAGENE, INC.) 11 July 1996 (11.07.96), page 4, line 30 to page 6, line 2, page 13, line 13 to page 22, line 14.	1-16
A	OKAYAMA et al. Bacteriophage lambda vector for transducing a cDNA clone library into mammalian cells. Molecular and Cellular Biology. May 1985, Vol. 5, No. 5, pages 1136-1142.	1-20
A	FLAMM et al. Expression of a cosmid containing the LCR, A-gamma, delta, and beta globin genes in mouse erythroleukemia cells. American Journal of Hematology. 1996, Vol. 53, pages 181-187.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

02 SEPTEMBER 1997

Date of mailing of the international search report
24 SEP 1997Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SCOTT D. PRIEBE

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12928

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HART et al. Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. Journal of Biological Chemistry. 29 April 1994, Vol. 269, No. 17, pages 12468-12474.	1-20

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*